2001

The Investigation of RNA Viruses using the Atomic Force Microscope

Michelle Moloney

*Biological Sciences, Cork Institute of Technology, Cork, Ireland.*

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The Investigation of RNA Viruses using the Atomic Force Microscope.

Michelle Moloney
The Investigation of RNA Viruses using the Atomic Force Microscope.

Michelle Moloney
Dedication:

To Mum and Ray.
Acknowledgements

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The Investigation of RNA Viruses using the Atomic Force Microscope.

A thesis presented to the Higher Education Training Awards Council
for the degree of
Doctor of Philosophy

By
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Cork.

June 2001

Research supervisor: Dr. Helen O'Shea.
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Abstract

The subject of viral entry into, and subsequent progeny virus egress, from cultured cells, has been extensively studied using numerous scientific techniques in fields ranging from cell culture to biochemistry to microscopy. The atomic force microscope (AFM) is a novel, developing instrument with unprecedented capabilities and this study was undertaken to observe enveloped Semliki Forest virus (SFV) and non-enveloped Theiler's Murine Encephalomyelitis virus (TMEV) entry into and egress from cultured cells using the AFM. Virus particle structure of both enveloped and non-enveloped viruses was also directly examined.

Cytopathic effect studies revealed that TMEV's are unable to produce progeny viruses in AT₃Neo cells. AFM analysis of TMEV entry into AT₃Neo cells revealed that the viruses are capable of infecting AT₃Neo cells. These results indicate a post-entry block in the TMEV replication cycle in AT₃Neo cells. AFM analysis of the egress of progeny viruses from cultured cells demonstrated that virally induced cytopathic effects can be visualised with the AFM. No consistent effects on cell viscoelasticity were observed during viral entry into and progeny virus egress from cultured cells. AFM analysis of virus particles demonstrated that antibody-coated (anti-capsid antibody and anti-envelope antibody) wafers are an effective means for the immobilisation of both enveloped and non-enveloped virus particles. Virus particles were successfully imaged and in all cases the virus particles appeared to collapse when they were immobilised.
### Abbreviations

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<thead>
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<tbody>
<tr>
<td>AFM</td>
<td>Atomic force microscope</td>
</tr>
<tr>
<td>BHK</td>
<td>Baby hamster kidney</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CPE</td>
<td>Cytopathic effects</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>dH₂O</td>
<td>Distilled water</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTH</td>
<td>Delayed type hypersensitivity</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>GMEM</td>
<td>Glasgow's minimal essential medium</td>
</tr>
<tr>
<td>h</td>
<td>Hours</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxidase</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>L</td>
<td>Leader</td>
</tr>
<tr>
<td>LSB</td>
<td>Low salt buffer</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium chloride</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NC</td>
<td>Non-coding</td>
</tr>
<tr>
<td>NCS</td>
<td>Newborn calf serum</td>
</tr>
<tr>
<td>NEC</td>
<td>Naturally empty capsid</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>NS</td>
<td>Non-structural</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PAM</td>
<td>Plaque assay medium</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate buffered saline containing 0.1% Tween</td>
</tr>
<tr>
<td>PFG</td>
<td>Paraformaldehyde-glutaraldehyde</td>
</tr>
<tr>
<td>p.i.</td>
<td>Post-infection</td>
</tr>
<tr>
<td>pi</td>
<td>Isoelectric point</td>
</tr>
<tr>
<td>PLP</td>
<td>Paraformaldehyde-lysine-periodate</td>
</tr>
<tr>
<td>Poly(A)</td>
<td>Polyadenylated</td>
</tr>
<tr>
<td>Ra</td>
<td>Roughness</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>sdH₂O</td>
<td>Sterile distilled water</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscope</td>
</tr>
<tr>
<td>SFV</td>
<td>Semliki Forest virus</td>
</tr>
<tr>
<td>ss</td>
<td>Single-stranded</td>
</tr>
<tr>
<td>STM</td>
<td>Scanning tunneling microscope</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscope</td>
</tr>
<tr>
<td>TMEV</td>
<td>Theiler's murine encephalomyelitis virus</td>
</tr>
<tr>
<td>TMEV-IDD</td>
<td>TMEV-induced demyelinating disease</td>
</tr>
<tr>
<td>TO</td>
<td>Theiler's original</td>
</tr>
</tbody>
</table>
TPB  Tryptose phosphate broth
TUNEL  Terminal deoxynucleotide transferase (TdT) mediated nick end-labelling
Chapter 1

Introduction
1.1: Theiler's Murine Encephalomyelitis Virus (TMEV).

1.1.1: History.

In 1933, Max Theiler discovered a young mouse with flaccid paralysis of the hind legs among a stock of normal mice in his laboratory (Theiler and Gard, 1934; Theiler, 1937). Theiler investigated mice with the symptom and discovered that they were suffering from a new viral disease which caused encephalomyelitis. This virus was later referred to as Theiler's Murine Encephalomyelitis Virus (TMEV). The original TMEV isolate is now referred to as the Theiler's Original (TO) virus and was the initial member of the first TMEV subgroup, the TO subgroup.

In 1940 Theiler and Gard discovered a second strain of TMEV, the GDVII strain, when they isolated the GDVII and FA viruses (Theiler and Gard, 1940). These two viruses were highly virulent when compared with the TO strain, and caused severe meningoencephalitis and death.

In 1952 Daniels et al., identified a new virus, called the DA (Daniels) virus which was similar to viruses of the TO strain. Daniels also provided the first description of demyelination induced by TMEV (Daniels et al., 1952). Since Theiler's discovery in 1933 a great deal of information regarding TMEV has been elucidated.
1.1.2: Virus family.

Theiler's murine encephalomyelitis viruses (TMEV's) belong to the virus family *Picornaviridae*. Picornaviruses are among the smallest ribonucleic acid (RNA)-containing viruses known. They are non-enveloped, spherical shaped, positive sense, single-stranded RNA viruses. The *Picornaviridae* comprise one of the largest and most important families of human and agricultural pathogens and are therefore of great economic and medical importance. *Picornaviridae* are currently divided into five genera; the rhinoviruses, the enteroviruses, the aphthoviruses, the cardioviruses and the hepatoviruses (Rueckert, 1996).

TMEV's were originally classified amongst the genus enterovirus. However, data obtained from nucleotide and amino acid sequence comparisons revealed that TMEV's are more closely related to the cardioviruses than the enteroviruses and they are now considered to be members of the cardiovirus genus (Ozden *et al.*, 1986).

1.1.3: TMEV strains.

TMEV's are a natural pathogen of mice; infection by the fecal-oral route results in an enteric infection (Jnaoui and Michiels, 1999). All strains of TMEV are grouped together because they are of one serotype and can be neutralized by the same polyclonal antiserum (Ohara and Roos, 1987). TMEV's are divided into two subgroups based on their differing biological and pathological properties and also on their reactions with monoclonal antibodies (Lipton, 1980; Lorch *et al.*, 1981;
Nitayaphan et al., 1985). Nucleotide variations in the 5' non-coding region and the L(leader)/P1 region have been implicated in the differing virulence abilities of the TMEV subgroups (Calenoff et al., 1990; Nash, 1991).

The GDVII subgroup strains, which include GDVII and FA, are neurovirulent and produce an acute fatal encephalomyelitis in all strains of mice examined (Ohara and Roos, 1987). Direct assessment of GDVII strain persistence is difficult because infected animals do not survive the acute stage of disease (Lipton, 1980). In GDVII subgroup infection the virus predominantly infects the gray matter, specifically the motor neurons in the cerebral cortex and ventral horns of the spinal cord. An inflammatory infiltrate of mononuclear cells accompanies the infection and the resulting encephalitis and cytolysis of neurons leads to death in 5-7 days (Dal Canto and Lipton, 1982; Martinat et al., 1999). It has been reported that the fatal outcome of GDVII infection may be due to the targeting of the infected neurons for apoptosis (Obuchi and Ohara, 1999; Anderson et al., 2000).

The second subgroup is the TO subgroup and it's members include the DA, WW, TO, BeAn and Yale viruses. Members of this subgroup are nominally avirulent but can express virulence, depending on the dose of the inoculum and the strain, age and immunological status of the recipient mouse (Theiler, 1937; Lipton, 1975). TO strains can establish a persistent infection and are associated with a biphasic disease. The initial or acute phase of the disease is characterized by a transient and mild neuronal poliomyelitis with virus replication occurring primarily
in the gray matter of the brain and spinal cord. Viral replication is prominent in neurons, but can also be detected in astrocytes and microglial cells (Anderson et al., 2000). The acute phase of disease lasts for 3-4 weeks and results in a flaccid paralysis. Animals surviving this phase progress towards chronic infection. At this stage the virus disappears from the gray matter and infects the white matter of the spinal cord, where it persists, mainly in the macrophage-microglial cells and, to a lesser extent, in oligodendrocytes (Martinat et al., 1999). Persistence of the virus in the white matter causes chronic inflammation and primary demyelination. The primary demyelination is exacerbated by the host's immune response and a severe and progressive demyelinating disease ensues (Friedmann and Lorch, 1985; Martinat et al., 1999; Anderson et al., 2000). The BeAn and WW strains can cause chronic disease without previously causing the acute phase (Dal Canto and Barbano, 1984; Lipton and Melvold, 1984).

There is 95% homology at the genomic level and a 90% homology at the protein level between the subgroups. Heterogeneity in the genomic regions, associated with neurovirulence and persistence, leads to the different biological properties of the TMEV subgroups. The phenotypic differences which exist between the two subgroups are summarised in Table 1.1 (Rodriguez et al., 1987).
<table>
<thead>
<tr>
<th>Parameters</th>
<th>TO subgroup</th>
<th>GDVII subgroup</th>
</tr>
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<tr>
<td>Members</td>
<td>DA, WW, BeAn, TO, Yale</td>
<td>GDVII, FA</td>
</tr>
<tr>
<td>Virulence</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Virus replication in CNS</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Acute disease</td>
<td>+/-</td>
<td>++</td>
</tr>
<tr>
<td>Chronic disease</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Virus persistence</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Plaque size (BHK-21 cells)</td>
<td>Small (1 mm)</td>
<td>Large (5 mm)</td>
</tr>
<tr>
<td>Ultrastructure (BHK-21 cells)</td>
<td>membranous</td>
<td>crystalline array</td>
</tr>
<tr>
<td>RNA synthesis <em>in vitro</em></td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td><em>in vitro</em> cell tropism</td>
<td>Neurons, glial cells, macrophages</td>
<td>Neurons</td>
</tr>
</tbody>
</table>

Table 1.1 Phenotypic differences which exist between the two TMEV subgroups. There is 95% homology at the genomic level and a 90% homology at the protein level between the subgroups. Heterogeneity in the genomic regions, associated with neurovirulence and persistence, leads to the different phenotypic properties of the TMEV subgroups.
1.1.4: Genetics of infection.

The outcome of infection by TMEV is dependant on the mouse strain, age and immunological status. The route of administration of the inoculum and the dose of inoculum given also affect the path of the infection (Lipton and Melvold, 1984).

The susceptibility of a mouse strain is under the control of several genes. Three loci which control viral persistence have been designated \textit{Tmevp1}, \textit{Tmevp2} and \textit{Tmevp3}. Susceptibility or resistance has been strongly linked to \textit{Tmevp1} which is the \textit{H-2D} gene of the major histocompatibility complex (MHC). The loci \textit{Tmevp2} and \textit{Tmevp3} are located on chromosome 10 (Bihl \textit{et al.}, 1999). Other genes implicated in influencing resistance/susceptibility to demyelination include an area on chromosome 3 near the \textit{Car-2} gene, an area on chromosome 6 near the \textit{Tcib} complex, an area on chromosome 18 near the \textit{Mbp} gene and a segment of chromosome 14 located between the \textit{Gnrh} and \textit{Glud} genes (Clatch \textit{et al.}, 1985; Rodriguez \textit{et al.}, 1986; Brahic and Bureau, 1998; Azoulay-Cayla \textit{et al.}, 2000; Drescher \textit{et al.}, 2000).

1.1.5: Virus structure.

TMEV is a single-stranded, non-enveloped, positive-sense RNA virus. The complete genomes of the GDVII, BeAn and DA viruses have been cloned and the nucleotide sequence determined (Pevear \textit{et al.}, 1987, 1988a and 1988b; Ohara \textit{et al.}, 1998). The TMEV genome is 8,098 nucleotides in length and has
**Figure 1.1** The TMEV genome. The genome is 8,098 nucleotides in length and has non-coding (NC) regions at the 5' and 3' ends. The 5' terminus is covalently linked to the VPg protein while the 3' terminus has a polyadenylated [Poly(A)] tail. The *P1* gene encodes the precursor protein P1 that is cleaved to produce the structural proteins VP1-4. Similarly, the *P2* and *P3* genes encode the precursor proteins 2A-C and 3A-D, respectively.
non-coding regions at the 5' and 3' ends (Figure 1.1). The 5' non-coding region is 1065 to 1069 nucleotides long and lacks a poly (C) tract. Covalently linked via a phosphodiester bond to the 5' terminus is the virus encoded protein VPg. A possible function for VPg is as a primer for RNA translation and it is therefore important for the initiation of viral replication. The 3' non-coding region is approximately 125 nucleotides long and has a polyadenylated tail. The 5' and 3' untranslated regions are known to play important roles in viral replication. The 500 nucleotides upstream of the authentic AUG start codon at 1,065 in the 5' untranslated region form a stable secondary structure that serves as an internal ribosome entry site and mediates cap-independent translation (Yamada et al., 1991; Lipton and Jelachich, 1997; Badshah et al., 2000).

The genome of TMEV is translated as a precursor polypeptide that is processed to yield all the proteins necessary for the viral life cycle. The genome consists of four core viral genes designated L, P1, P2 and P3. The L gene is the first to be translated and it codes for the L (leader) protein. The P1 gene is translated second and codes for the four structural proteins (VP1-4) of the virus. The P2 gene is translated next and codes for three viral proteins 2A-C. Finally the P3 gene which codes for four viral proteins (3A-D) is translated.

The TO subgroup strains of TMEV have an alternative translation initiation codon within the L coding region at nucleotide 1,079, in addition to the authentic initiation site. This alternative initiation site is out-of-frame with the polyprotein
and is used to translate a small 17 kDa protein, designated L*. The GDVII subgroup strains of TMEV do not contain the L* initiation site and therefore are not capable of directing the synthesis of L*. This protein is important for virus growth in particular cell types (e.g. macrophages) and plays a critical role in TO subgroup persistence. L* is associated with membranes of the infected cell, suggesting that it may interact with the immune system and thereby mediate the viral-induced demyelinating disease (Chen et al., 1995; Obuchi and Ohara, 1999; Badshah et al., 2000; Obuchi et al., 2000; Van Eyll and Michiels, 2000).

The three-dimensional structures of GDVII, BeAn and DA viruses have been determined at approximately 3 Å resolution by x-ray crystallography (Figure 1.2) (Grant et al., 1992; Luo et al., 1992 and 1995). A protein shell (capsid) encapsidates the ssRNA genome. The entire virus structure is 20-30 nm in diameter. The virions have an icosahedral symmetry i.e. they have a 60-subunit protein shell with 20 triangular faces, 5 at the top, 5 at the bottom and 10 around the middle. The icosahedral capsid is formed by the packing of wedge-shaped eight-stranded anti-parallel β-barrels (Grant et al., 1992). Mature virions possess three major structural polypeptides, VP1 (37 kDa), VP2 (34 kDa) and VP3 (27 kDa), and a smaller fourth major polypeptide, VP4 (6 kDa) (Lipton and Friedmann, 1980; Luo et al., 1992). VP1, VP2 and VP3 form the eight-stranded, anti-parallel β-barrels that subsequently form the capsid. The sequences that connect the β-strand form loops that are found on the exterior of the virion. These
Figure 1.2 The molecular surface of TMEV. Resolved by x-ray crystallography and viewed along the two-fold axis (Image by Stephen Spencer, ©1995 Academic Press).
surface loops contain the major antibody neutralising sites for TMEV. VP4 is found exclusively lining the interior surface of the capsid in close association with the RNA core (Rueckert, 1996; Lipton and Jelachich, 1997; Hertzler et al., 2000).

1.1.6: Virus infection and replication.

The initial event in infection is attachment of the virion to specific receptor units embedded in the plasma membrane. Structural studies indicate that the cardiovirus pit, a deep depression on the surface of the virion between VP1 and VP3 along the two-fold axis, is involved in receptor attachment (Lipton and Jelachich, 1997; Hertzler et al., 2000). Both persistent and neurovirulent TMEV strains bind to a 34-kDa glycoprotein, which is abundant in neurons and BHK-21 cells, the latter being used to grow the virus in vitro (Jnaoui and Michiels, 1999). The binding of persistent TO subgroup strains is dependent on the interaction with both a sialyl moiety and the protein surface of the receptor, while neurovirulent GDVII subgroup strains are only dependent on the interaction with the protein surface of the receptor (Zhou et al., 2000). Different TMEV's strains, therefore, bind to the same cellular glycoprotein (34 kDa) receptor but in different ways.

The viral uncoating proceeds via receptor-mediated endocytosis. This is a process whereby the virus-receptor complexes on the plasma membrane cluster at clathrin-coated pits and are subsequently internalized by invagination. Acidification of the resulting clathrin-coated vesicles leads to the loss of VP4 and
Figure 1.3 TMEV infection and replication. The cardiovirus pit of the TMEV particle attaches to the cellular glycoprotein receptor and receptor-mediated endocytosis results. The viral RNA is transferred into the cytoplasm where it is translated to produce the proteins required for replication and new progeny capsids and transcribed to form progeny genomes. The progeny genomes and capsids are assembled into progeny virions which are released by host cell lysis.
to the formation of a pore through which the viral RNA can be transferred into the cytoplasm (Figure 1.3).

The first step in the replication of all positive-sense RNA viruses is the translation of the infecting RNA. For reviews of the viral replication cycle see Levy et al., 1994a, Jakob and Roos, 1996 and Rueckert, 1996. The incoming RNA strand directs the synthesis of a polyprotein using the host’s ribosomes and other protein synthesizing machinery (Figure 1.4). This polyprotein is cleaved into segments while still in the process of synthesis. Cleavage results in the formation of three precursor proteins, P1, P2, and P3, P1 being the first to be released and P3 the last. Cleavage is mediated by proteinases encoded in the polyprotein.

Protein P1 is a precursor of the structural proteins of the virion. It is cleaved to produce VP4, VP2, VP3 and VP1. The P1 region of the virus genome is associated with neurovirulence and the rate of virus replication (O’Shea et al., 1997). P2 is cleaved to produce three non-structural proteins 2A, 2B and 2C. Proteins 2B and 2C are involved in RNA synthesis, while protein 2A is involved in the shut-off of host protein synthesis. P3 can be cleaved by two separate mechanisms. One mechanism is a concentration-independent self-cleavage that yields (a) a proteinase, 3C; (b) a protein, 3AB, which is involved in initiating RNA synthesis; and (c) an RNA polymerase, 3D, which can elongate a primer RNA bound to a template RNA. The second mechanism is a bimolecular cleavage
Figure 1.4 Translation of the TMEV genome into its respective proteins. VP1, 2, 3 and 4 are the structural proteins; 2A-C and 3A-D are the nonstructural proteins; 3AB is involved in initiating RNA synthesis, 3C is a proteinase and 3D is a RNA polymerase.
involving one P3 molecule and the 3C proteinase generated by cleavage of another P3 precursor molecule. The first step in synthesis of new viral RNA is to copy the incoming genomic RNA to form complementary minus-strand RNA, which then serves as a template for synthesis of new plus strands. During the early steps of replication, newly synthesised plus-stranded RNA molecules are recycled to form additional replication centers until, with an ever-expanding pool of plus-stranded RNA, a greater and greater fraction of the plus-stranded RNA in the replication complex is packaged into virions.

Assembly of new virions begins with the cleavage of two intersubunit bonds within polypeptide P1. This is catalysed by protease 3C or 3CD and is followed by substantial structural rearrangements which result in the formation of 5S capsid protomers containing VP0, VP3 and VP1. Association of 5 such protomers to form a 14S pentamer is followed by assembly into a noninfectious RNA-containing provirion which is also known as an NEC (or naturally empty capsid). Whether pentamers condense around RNA or RNA is threaded into pentamers is still a subject of debate.

Provirions are not infectious. Formation of the infective virion requires a "maturation cleavage" in which most of the VP0 chains are cleaved to form the mature four-chain subunits (VP4, 2, 3, and 1). Completed virus particles are ultimately released by infection-mediated disintegration of the host cell.
The time required for a complete multiplication cycle, from time of infection to completion of virus assembly takes from 5 to 10 hours.

1.1.7: In vitro characteristics.

Theiler's viruses were adapted to grow in cell culture by blind passages. TO strains were readily grown in primary mouse tissues and cultures derived from embryos. TMEV is commonly propagated in BHK-21 and L2 cell cultures (Graves et al., 1986; Yamada et al., 1991). TMEV has also been propagated in numerous other cell cultures e.g. Schwann cell cultures (Frankel et al., 1986), brain macrophage cell cultures (Levy et al., 1992) and mixed glial cell cultures (O'Shea et al., 1997).

TMEV lytically infects neurons and oligodendrocytes and persistently infects astrocytes and macrophages (Graves et al., 1986; Aubert et al., 1987). The GDVII and FA strains replicate more readily and to a higher titre in cell culture compared to the TO subgroup strains. GDVII and FA strains form large crystalline arrays of virions within the cytoplasm of infected cells and are readily released upon cell lysis. In contrast, TO viruses arrange themselves in a single file between the two layers of membranes in the cytoplasm of infected cells and remain trapped within the membranes upon lysis of infected cells (Lipton and Friedmann, 1980; Frankel et al., 1986; Obuchi and Ohara, 1999).
In vitro passage of TO viruses results in a marked attenuation of their virulence, however, they do retain their capacity to induce disease. L929 cells can be persistently infected with the DA virus strain. Studies have shown that only a small number of cells in the cultures contained infectious virus (Friedmann and Lorch, 1985).

1.1.8: Physical properties.

Since TMEV's do not have an envelope, they are more resistant than lipid-containing viruses to chemicals and physical agents. TMEV's are insensitive to chloroform, ether, nonionic detergents (e.g. Tween-80) and the ionic detergent sodium dodecyl sulfate. TMEV's are inactivated by 0.3% formaldehyde and 0.1 N HCl. They are rapidly destroyed at temperatures over 50°C and lose some infectivity upon lyophilization. Purified virions can be stored for long periods of time at -70°C without loss of infectivity, but slowly lose infectivity on storage at -20°C. TMEV's are stable at low pH as they encounter acidic conditions in the stomach and they are stable over the entire pH range from 3 to 9.5.

1.1.9: Immune response.

The timing of certain immune components is important in the protection against TMEV infection. Initial host immune responses are fast and non-specific and include interferon (IFN) α/β and natural killer (NK) cells which are essential in rapidly limiting the early spread of the infection in the gray matter (Monteyne et al., 1997).
During the first week post-infection (p.i.), mice mount a virus-specific humoral immune response. This response peaks by one month p.i. and is sustained thereafter (Lipton and Jelachich, 1997). Neutralising antibodies are important in limiting viral spread both during early gray matter disease and during the late white matter infection (Welsh et al., 1987; Monteyne et al., 1997). Antibody epitopes include three epitopes located in VP1 protein (VP1_{12-25}, VP1_{146-160} and VP1_{262-276}), two in VP2 (VP2_{2-16} and VP2_{165-179}) and one in VP3 protein (VP3_{24-37}) (Cameron et al., 2001). Immunisation with VP1 and VP2 fusion proteins prior to viral infection, but not VP3, can result in protection from subsequent development of demyelination (Kim and Palna, 1999).

TMEV infection also results in the production of a virus-specific cellular immune response. This involves both the T helper (CD4^+) and T cytotoxic (CD8^+) subsets of the T lymphocytes. T cell proliferative responses and delayed type hypersensitivity (DTH) responses (mediated by CD4^+ T cells) appear by two weeks p.i. and remain elevated for at least six months. DTH and T cell proliferative responses are directed to VP1_{233-250}, VP2_{74-86} and VP3_{24-37}, one on each major external capsid protein (Lipton and Jelachich, 1997; Kim and Palna, 1999; Cameron et al., 2001). Functional roles of the CD4^+ T cells include aiding the B lymphocytes to mount the humoral immune response and CD4^+ Th1-type cells favour a protective cytotoxic T cell (CTL) response (Monteyne et al., 1997). The protective role of virus-specific CTL’s is probably invoked by limiting virus-
infected cells (Kim et al., 2000). The main epitope of CTL's is located on the VP2 protein (VP2_{121-130}) (Cameron et al., 2001).

Several additional components of the immune system such as macrophages, chemokines, cytokines, TGF-β2 and CD40L are also linked to the TMEV induced immune response (Drescher et al., 2000a and 2000b; Kim et al., 2000; Murray et al., 2000; Van Eyll and Michiels, 2000). Although mice mount virus-specific humoral and cellular immune responses, TMEV evades immune clearance to persist at low levels in the central nervous system (CNS) (Lipton and Jelachich, 1997).

1.1.10: Viral persistence.

The roles of the immune response during TMEV infection are to limit virus replication, eliminate virus and suppress the acute encephalitis due to viral cytopathic effects. Escape of the virus from the immune system results in viral persistence.

Considerable evidence favors the macrophage as the predominant target cell for TMEV persistence, since the majority of the virus load resides in these phagocytic cells (Lipton and Jelachich, 1997; Obuchi et al., 2000; Van Eyll and Michiels, 2000).
Analysis of chimeric viruses constructed by exchanging the capsids of neurovirulent and persistent virus strains revealed that the capsid bears the main determinants of persistence and neurovirulence (Peaver et al., 1988c; Calenoff et al., 1990; Fu et al., 1990; Lipton et al., 1991; Tangy et al., 1991; Jarousse et al., 1994; O'Shea et al., 1997; Jnaoui and Michiels, 1999; Martinat et al., 1999). In particular, the leader and P1 sequences encoding the capsid proteins have been identified as the determinant(s) for viral persistence. Persistence has been shown to require a specific capsid conformation, involving the VP2 puff and VP1 loops, which may influence viral persistence through virion receptor binding or attachment to host cells (Lipton and Jelachich, 1997).

1.1.11: Demyelination.

Demyelination takes place a month or more post-infection. Demyelination occurs mainly in the white matter of the spinal cord and it is characterised by extensive destruction of myelin sheaths with preservation of the axons. This results in dissipation of nerve impulses which essentially causes a breakdown in communication between the brain and the muscles of various organs, thereby resulting in paralysis and death.

Although the mechanisms of TMEV induced demyelinating disease (TMEV-IDD) are not fully understood, several studies have suggested that host immune responses against the virus plays a key role. The major factor in myelin destruction seems to be virus-specific inflammatory CD4\(^+\) Th1-type cells directed
against the VP1 and VP2 capsid proteins. The development of TMEV-IDD is therefore a T-cell mediated process, correlating with high levels of TMEV-specific DTH and viral persistence in the CNS. The CD4+ T-cell responses target CNS persistent virus leading to macrophage-mediated bystander destruction of CNS myelin. The chronic stage of TMEV-IDD also involves the activation of autoreactive CD4+ T cells specific for a variety of myelin epitopes which are induced via epitope spreading (Cameron et al., 2000; Haynes et al., 2000; Katz-Levy et al., 2000; Kim et al., 2000; Koh et al., 2000; Neville et al., 2000).

Recent reports indicate that proinflammatory cytokines are involved in the neuropathogenesis of CNS disease (Theil et al., 2000) and that the anti-myelin response that emerges during the chronic phase of infection is functionally active (Dal Canto et al., 2000).

The role of TMEV in the induction of autoimmune disease resulting in demyelination has important implications when studying the aetiology of the human demyelinating disease, multiple sclerosis (Nash, 1991).
1.2: Semliki Forest Virus (SFV).

1.2.1: History.

Semliki Forest virus (SFV) was first isolated by Smithburn and Haddow from mosquitoes captured in the Semliki Forest in Uganda in 1944. SFV is serologically related to Eastern equine, Western equine and Venezuelan equine encephalitis viruses. It has been isolated in Africa, India and Southeast Asia. SFV infects mosquitoes, small rodents and man (Mathiot et al., 1990).

1.2.2: Virus Family.

SFV belongs to the Togaviridae, genus alphavirus (Porterfield et al., 1978; Schlesinger and Schlesinger, 1996). The togavirus family contains two genera, the alphaviruses and the rubiviruses (Levy et al., 1994b). The family Togaviridae was originally much larger and included flaviviruses, pestiviruses, and other viruses that had not been well characterised. More detailed knowledge of the genome, structure and replication strategy of many of these viruses led to the establishment of the family Flaviviridae which now seems almost as diverse as the Togaviridae once was (Schlesinger and Schlesinger, 1996).

1.2.3: SFV strains.

All strains of SFV are lethal when administered to neonatal mice (Smyth et al., 1990). However, SFV strains can be divided into two subgroups, based on their differing virulence in adult and weanling mice. Studies have shown that the virulence of SFV strains is controlled by rapidity of multiplication in the CNS,
leading to a lethal threshold of damage, rather than differential cell tropism or cell death mechanism (Glasgow et al., 1997).

The first subgroup is the L10 subgroup. The L10 virus is virulent and causes lethal encephalitis in mice of all ages, leading to death of the animals in a few days (Tuittila et al., 2000). L10 virulence is due to its ability to cause extensive neuronal damage in the CNS (Atkins et al., 1990). This damage exceeds the lethal threshold of damage to neurons and takes place before immune-intervention can occur (Gates et al., 1985). A second neurovirulent strain, SFV4, has been produced by transcription of the pSP6-SFV4 infectious clone of the L10 stain (Atkins et al., 1999).

The second subgroup contains the avirulent A7 and M9 virus strains (Johnston and Peters, 1996). The A7 strain was first isolated in mosquitoes in Mozambique in 1959 (McIntosh et al., 1961). The M9 strain is an avirulent mutant of the L10 virus. Avirulent strains induce an acute disease resulting in paralysis. Persistence of the virus leads to a chronic disease state, resulting in autoimmune demyelination by a mechanism which involves infection of oligodendrocytes (Sheahan et al., 1983; Gates et al., 1985). A7 can not only traverse the blood-brain barrier via infection of vascular epithelial cells, but can also cross the placenta and it has been shown to produce 100% fatality in mouse embryos (Atkins et al., 1982 and 1999). The replication of A7 in mouse neurons is restricted by the age of the infected animal. This restriction is not associated with
the maturity of virus-specific immune responses but probably reflects age-related changes in neurons (Fazakerley et al., 1993). An additional avirulent strain, A7(74), has been derived from the A7 strain by further selection for avirulence (Atkins et al., 1995). M9 shows sustained multiplication in the brains of infected mice. It produces paralysis in 35%, and death in 8% of infected mice. Demyelination occurs in 94% of the surviving mice (Atkins and Sheahan, 1982).

1.2.4: Virus structure.

The intact SFV is enveloped. The enveloped virion is spherical, 65-70 nm in diameter with icosahedral symmetry and a T=4 lattice (Figure 1.5). The internal nucleocapsid is also spherical, 35-42 nm in diameter with an icosahedral symmetry and a T=3 lattice. The SFV capsid consists of 240 monomers of a single species of protein (Levy et al., 1994b). The molecular weight of SFV core protein is 33 kDa (Kaariainen et al., 1969).

Inside this capsid lies the viral genome. The SFV genome is composed of a 49S single-stranded (ss), positive-sense, non-segmented RNA molecule, which is approximately 11.7 kb in length (Schlesinger and Schlesinger, 1996; Atkins et al., 1999) (Figure 1.6). The genome makes up 4-8% of the total weight of viral particle and 38% of the core (Kaariainen et al., 1969). The RNA genome is polyadenylated at the 3' end and capped with 7-methylguanosine at the 5' end.
Figure 1.5 The molecular surface of SFV (Fuller et al., 1995). The viral envelope (blue) is spherical, 65-70 nm in diameter with icosahedral symmetry and a T=4 lattice. The envelope membrane contains 80 spike protein trimers. The internal nucleocapsid (yellow) is also spherical, 35-42 nm in diameter with an icosahedral symmetry and a T=3 lattice.
Figure 1.6 The SFV genome. The genome consists of a single-stranded (ss) positive-sense ('+') 49S RNA. The genome which is approximately (~) 11.7 kb in length is polyadenylated at the 3' end and methylguanosine capped at the 5' end. A 26S subgenomic RNA is contained within the genome.
(Levy et al., 1994b). The 49S genome contains a 26S subgenomic RNA which codes for the viral structural proteins.

The nucleocapsid is surrounded by the envelope, which is a lipid bilayer derived from host cell plasma membrane. The envelope membrane contains 240 copies of each of two viral transmembrane proteins, E1 (49 kDa) and E2 (52 kDa), and of an extrinsic envelope protein, E3 (10 kDa) (Mancini et al., 2000; Mancini and Fuller, 2000). The E1, E2 and E3 viral proteins form spike protein trimers in the viral envelope. Each spike trimer consists of three copies of each viral protein (Schlesinger and Schlesinger, 1996; Shome and Kielian, 2001). Proteins of the viral envelope have regions that are rich in hydrophobic amino acids (e.g. leucine, isoleucine, valine, phenylalanine) which are always embedded in the plasma membrane. The hydrophilic regions form the exterior spikes. As the lipids of the virion envelope are derived from host cell membranes they are therefore specified by host cell enzymes. Similarly, the addition of sugar residues to viral glycoproteins appears to involve host cell enzymes (Levy et al., 1994b).

As viral envelopes generally lack host cell surface antigens, the viral envelope proteins are thought to displace all host cell proteins from the regions of the plasma membrane involved in virion maturation. Insertion of viral proteins into the cell membranes is responsible for a wide variety of biological effects on the host cell e.g. hemagglutination and for SFV this is determined by the glycoprotein E1 (Levy et al., 1994b).
1.2.5: Virus infection and replication.

Replication of SFV occurs in the cytoplasm and takes approximately 4 hours (Schlesinger and Schlesinger, 1996). The first step in the replicative process is viral entry into the target cell (Figure 1.7). SFV enters cells initially by viral envelope spike protein binding to the host cell plasma membrane. The E2 protein mediates host-cell recognition and attachment (Mancini et al., 2000). The H2 and HLA histocompatibility antigens were reported to be the receptors for SFV but their presence on the cell surface is not essential for infectivity (Schlesinger and Schlesinger, 1996). The enveloped virion is then internalised into the cell via an endosome.

The next step in viral entry is the movement of the viral genome from the endosome-contained virion into the host cell cytoplasm. This occurs via fusion of the viral envelope with the endosome membrane. The low pH (usually less than 6.2) of the endosome triggers conformational changes in the E1 spike protein subunit, which exposes a previously hidden hydrophobic region of the protein termed the fusion peptide. These conformational changes are strongly cholesterol and sphingolipid-dependant (Chatterjee et al., 2000). The fusion peptide inserts into the endosome bilayer so that the spike protein is interacting with both the target membrane and the virus membrane. This dual membrane interaction drives the mixing of the outer leaflets of the two membranes, a process termed hemifusion. Complete fusion occurs when the mixing of the inner
Figure 1.7 The replication cycle of SFV. The virus attaches to the host cell membrane and enters the cell in an endosome. The virus envelope and the endosome membrane fuse together, thus releasing the viral genome into the cell cytoplasm. The 49S viral RNA is translated to produce the non-structural proteins, which subsequently become involved in the transcription of the viral genome. The genome is transcribed to produce both the 26S and 49S viral RNAs. The 26S RNA is translated to produce the structural proteins which associate with the newly synthesised '+' 49S RNA to form the viral progeny nucleocapsids. The viral envelope proteins embedded in the host cell membrane become associated with the nucleocapsid and the progeny virus buds from the host cell membrane to produce the mature virion.
membrane leaflets occurs leading to the release of the virus genome into the cytoplasm (Kielian et al., 1996; Ahn et al., 1999; Gibbons et al., 2000; Shome and Kielian, 2001).

The second step in the replicative process is the translation of the 49S genomic RNA, which yields a polyprotein containing 4 non-structural (ns) proteins (Figures 1.7 and 1.8). An autoproteolytic activity present in nsP2 is responsible for some of the cleavages necessary to release all four ns polypeptides from the polyprotein precursor (Levy et al., 1994b). The nsP1 is a methyl- and guanylyltransferase, nsP2 can act as a proteinase (i.e. cleavage of polyprotein) or as a nucleoside triphosphatase. The nsP3 is a phosphoprotein which is proposed to function with nsP1 in anchoring the replication complex proteins to cytoplasmic membrane structures (Tuittila et al., 2000).

The four ns polypeptides catalyze replication of the 49S genomic RNA as well as transcription of a 26S subgenomic mRNA (Figures 1.7 and 1.8). RNA synthesis initiates at the 3' terminus of the 49S RNA, and this process presumably involves both the 19 conserved nucleotides adjacent to the poly(A) tract as well as another 51 conserved nucleotides near the 5' terminus. The 49S genomic RNA serves as template for negative-sense RNA synthesis. The resulting full-length negative-sense RNA serves as a template for the synthesis of two types of positive-sense RNAs. Whereas initiation at the 3' end of the negative sense strand yields a full length 49S positive-strand progeny, initiation within the
Figure 1.8 Transcription and translation of SFV genome. The positive (‘+’) sense single-stranded 49S RNA genome is translated into a polyprotein, which is subsequently cleaved to produce the non-structural (ns) proteins; nsP1 is a methyl and guanylyltransferase; nsP2 can function as a proteinase or as a nucleoside triphosphatase; nsP3 is a phosphoprotein. The positive (‘+’) sense single-stranded 49S RNA genome is also transcribed to provide a negative (‘-’) sense single-stranded 49S RNA template. This template can be further transcribed to produce the positive (‘+’) sense single-stranded 49S RNA progeny genomes or the positive (‘+’) sense single-stranded 26S RNA subgenome. The 26S RNA subgenome is translated and the resulting polyprotein cleaved to produce the viral structural proteins; 6K functions late in the viral assembly pathway; C is the capsid protein, p62 which is further cleaved to produce the envelope proteins E2 and E3; E1 is also an envelope protein.
'+' 49S RNA ----> translation
Polyprotein

nsP1, nsP2, nsP3, nsP4

transcription

'-' 49S RNA

transcription

'+' 26S subgenome

transcription

't+' 49S RNA

transcription

'+' 26S subgenomic

Polyprotein

6K, C, p62, E1

E2, E3

translation
conserved junction region between the non-structural and structural gene clusters leads to the formation of 26S mRNA (Levy et al., 1994b).

The 26S subgenomic RNA carries the genetic information for the virion structural proteins (Figure 1.8). Firstly, a 6K protein, the absence of which results in a dramatic reduction in virus release, therefore 6K exerts its function late in the assembly pathway possibly during virus budding (Liljestrom et al., 1991). The 26S RNA also codes for C, the capsid protein and p62 which is cleaved to form E2 a virulence determinant (Santagati et al., 1995) and E3, both of which are envelope proteins. Finally the 26S RNA codes for E1, the third envelope protein.

The 26S RNA is produced intracellularly early during infection and codes for the most highly expressed viral proteins. The large genomic RNA supplies the smaller amounts of enzymatic products required. The SFV subgenome contains a translation enhancer region in the coding part of its RNA. The enhancer increases the translation of the viral structural genes approximately ten-fold and thereby plays a key role in virus assembly. This translation enhancer represents an adaptation to the host cells defence and is only functional in the virus-infected cell (Sjoberg and Garoff, 1996).

Synthesis of both positive- and negative-sense RNA increases during the first several hours after infection. Negative-sense RNA synthesis then stops, whereas synthesis of the 49S and 26S positive-sense RNAs continues at a nearly
constant rate. Because most of the 49S RNA is sequestered in nucleocapsids, the ratio of polysome-bound 26S mRNA to 49S RNA can be as much as 10:1. The translation of the 26S subgenomic mRNA is a result of cytoplasmic translation on free ribosomes.

Once all the individual components of the virus are manufactured, the newly synthesised ss positive-sense 49S RNA molecules are packaged into the capsid protein, thus forming the nucleocapsid. The amino terminus of the capsid protein is relatively rich in basic amino acids plus proline, and sequences near (but not at) the 5' terminus of the 49 S RNA determine the specificity of the RNA-capsid protein association (Levy et al., 1994b).

The final stages of replication occur at the plasma membrane, where the viral nucleocapsid, the glycoprotein spikes, and the lipid bilayer are brought together to form the mature virion (Figure 1.7). This process involves the association of the cytoplasmic tail of E1/E2 heterodimers with the nucleocapsid (Shome and Kielian, 2001). Mutations in the E1/E2 dimer reduce the efficiency of lateral spike protein interactions required for virus budding (Duffus et al., 1995). Two amino acids (leucine 401 and tyrosine 399) in the cytoplasmic tail of the spike protein form a motif that is required for viral budding (Skoging-Nyberg and Liljestrom, 2000). Virus budding from the plasma membrane appears to be initiated by interactions between the nucleocapsid and a specific domain in membrane-bound E2 that is exposed to the cytoplasm. Conversion of p62 to E2 and E3 may
promote this interaction (Levy et al., 1994b). The budding of SFV from the host cell requires cholesterol to prevent spike protein degradation at the cell plasma membrane (Lu and Kielian, 2000).

1.2.6: In vitro characteristics.

SFV4 and A7 exhibit similar cell tropism in the CNS of adult mice and similar molecular properties. However, they differ in the severity and rate of development of cytolytic damage (Atkins, 1983; Balluz et al., 1993). A7 and SFV4 cause apoptotic death in BHK-21 cells, oligodendrocytes of glial cell cultures, AT₃ cells and CHO cells (Scallan et al., 1997; Mastrangelo et al., 2000). For cerebellar neuron cultures, virus-induced death is due to necrosis. SFV4 multiplies to a higher titre in cultured neurons than the avirulent A7 strain (Glasgow et al., 1997).

L10 multiplies to a higher titre than M9 and A7, while M9 multiplies to a higher titre than A7 (Gates et al., 1985). The molecular and host range properties of the L10 strain have been compared to those of the A7 strain. No difference could be detected between the two strains in adsorption, nucleocapsid synthesis, protein synthesis, ratio of 49S:26S RNA, particle infectivity, interferon induction and susceptibility, or defective interfering particle production (Atkins, 1983). A7 did, however, show lower total RNA synthesis than L10 in BHK-21, G26-24 (oligodendroglioma) and C1300 (neuroblastoma) cells. Cytopathogenicity of A7
was reduced compared to L10 in C1300 cells but not in G26-24 cells (Atkins, 1983).

1.2.7: Physical properties.
As a result of the high lipid content of the SFV envelope, virions are very sensitive to ether and detergents, they are also inactivated by reducing agents, slightly elevated temperatures and certain salts (Levy et al., 1994b). Virions lose infectivity after treatment with sodium deoxycholate. Nonidet P40 destroys the integrity of the lipid-containing envelope membrane of SFV. Tween-ether treatment also destroys the infectivity of SFV (Kaariainen et al., 1969).

1.2.8: Immune response.
Humoral immunity is important for protection against viral infection and neutralisation of extracellular virus, but clearance of virus from infected tissues is thought to be mediated solely by cellular immunity. However, in a SCID mouse model of persistent alphavirus (Sindbis virus) encephalomyelitis, adoptive transfer of hyperimmune serum resulted in clearance of infectious virus and viral RNA from the nervous system, whereas adoptive transfer of sensitised T lymphocytes had no effect on viral replication. Three monoclonal antibodies to two different epitopes on the E2 envelope glycoprotein mediated the viral clearance. Thus, antibody can mediate clearance of alphavirus infection from neurons and subsequently prevent persistence by restricting viral gene expression (Levine et al., 1991). However, anti-myelin antibodies and B cells in
the brains of mice following active SFV infection can induce CNS white matter injury (Smith-Norowitz et al., 2000). This indicated both a protective and pathologic role of the immune response during SFV infection.

1.2.9: Viral persistence.
The avirulent strains of SFV can persist in infected animals and it is this persistence which leads to the chronic stage of disease. The genetic loci responsible for the natural avirulence of the A7(74) strain reside entirely in the non-structural genome and they mainly derive from changes in the replicase complex nsP3 gene (Tuittila et al., 2000). The A7 E1 glycoprotein has been shown to persist in the cerebral capillaries, while E2 has been shown to persist in the cytoplasm of neurons, particularly in the hippocampal areas and glia in the cerebellum (Khalili-Shirazi et al., 1988). The persistent presence of viral antigens has also been demonstrated in astrocytes and oligodendrocytes. The presence of life-long anti-SFV antibody in the sera of animals after SFV infection could be due to the persistence of viral antigens, acting as constant stimuli to the immune system (Khalili-Shirazi et al., 1988).

1.2.10: Demyelination.
Mice surviving infection by avirulent strains of SFV show virus-induced demyelination. This demyelination occurs in discrete areas of the CNS called plaques and avirulent SFV has therefore been suggested as a model for human
demyelinating diseases such as multiple sclerosis (Illavia and Webb, 1988; Atkins et al., 1995).

The adherence of inflammatory cells to the endothelium and migration into the CNS are essential steps for the development of autoimmune demyelination. This adhesion and migration is dependent on the expression and upregulation of cellular adhesion molecules. In SFV A7 infection, the expression of $\alpha_4$-integrin VLA adhesion molecule plays a major role in demyelination due to cell recruitment (Smith et al., 2000).

Oligodendrocytes are the primary CNS target of the avirulent SFV strains (Gates et al., 1984). Direct virus-induced injury to oligodendrocytes leads to the initiation of an acute inflammatory reaction, which includes lesions of primary demyelination (Sheahan et al., 1983). The immune system responds by phagocytosing the resulting myelin debris from infected oligodendrocytes and by presenting this debris in the form of antigens to T lymphocytes (Atkins et al., 1990). The T cells become activated and drive the immune system to target myelin components as foreign antigens. This serves to exacerbate the demyelination induced originally by the virus. In 1993 Subak-Sharpe et al., showed that the variety of T cell responsible for this immune mediated demyelination are the cytotoxic CD8$^+$ T lymphocytes.
In 2000, Lawson showed that there was a sequence similarity between the viral E2 protein (residues 115-129) and myelin oligodendrocyte glycoprotein (residues 18-32). Thus, the occurrence of molecular mimicry between viral antigens and self-determinants may also contribute to the pathogenic mechanism of SFV resulting in demyelination.
1.3: Atomic Force Microscopy.

1.3.1: History of microscopy.

The human eye has the ability to detect an object 0.2 mm in diameter. Most cellular structures are therefore too small to be seen by the unaided eye. The invention of the light microscope enabled scientists to see enlarged images of cells for the first time and the first generally useful light microscope was developed in 1590 by Z. Janssen and his nephew H. Janssen (Prescott et al., 1998; Becker et al., 1999).

Since then light microscopes have been improved and modified, right up to the present day. Other early microscopists include A. Van Leeuwenhoek, a Dutch shopkeeper, who is generally regarded as the father of microscopy, used a simple single-lense microscope while other microscopists, such as Hooke, employed primitive compound microscopes. The latter were subject to chromatic aberration because only simple lenses were employed. Following the introduction of improved achromatic microscopes by Lister in 1827, light microscopy has developed to an extremely high standard (Smith and Wood, 1996; Becker et al., 1999).

Today the instrument of choice for light microscopy uses several lenses in combination and is therefore called a compound microscope. There are many forms of light microscopy, all of which are variations on a general theme, examples include phase contrast microscopy, confocal microscopy and
fluorescence microscopy (Smith and Wood, 1996; Prescott et al., 1998; Becker et al., 1999).

The next revolutionary invention in the field of microscopy was the electron microscope. The electron microscope is of much more recent vintage than the light microscope and it dates from the early 1930's. Electron microscopy triggered a revolution in the exploration of cell structure and function, and ultimately in the way we think about cells. Two basic designs exist in electron microscopy, the transmission electron microscope (TEM) and the scanning electron microscope (SEM). The TEM forms an image by measuring the differing extents of electron transmission through a specimen. The SEM, which is a relatively recent development, generates an image by scanning the specimen with a beam of electrons. The TEM was a huge improvement over the light microscope and it has led to an enormous advance in the knowledge of the ultrastructure of cells. The SEM, on the other hand, gives a sense of depth to biological structures thereby allowing surface topography to be studied (Smith and Wood, 1996; Becker et al., 1999).

Light and electron microscopy are direct imaging methods, in that they use photons and electrons respectively, to produce actual images of the specimen. The most recently developed microscopes, the scanning probe microscopes, are indirect imaging methods. Scanning probe microscopes measure small forces active at the specimen surface. They use a small probe, positioned very close to
the specimen surface, which is scanned across the surface, taking measurements at regular intervals. This produces a surface profile of the molecular structure of the surface (Smith and Wood, 1996; Becker et al., 1999). One of the first scanning probe microscopes was the scanning tunneling microscope (STM) which was built as recently as 1982. Operation of the STM is based on a quantum effect that as two conductors are brought together, electrons will 'tunnel' through the narrow insulating gap between them. A small change in the distance between the probe and the specimen produces a relatively large change in the tunneling current. The changes in current can be measured as the probe scans the specimen and are used to build up the image (Smith and Wood, 1996). In 1986 Binnig et al., invented a new type of scanning probe microscope, the atomic force microscope (AFM) which is now emerging as an invaluable tool for biologists.

1.3.2: Comparison of microscopes.

Microscopes can be compared on the basis of resolution, sample preparation and range of specimens that can be analyzed. Resolution is the ability to separate or distinguish between small objects that are close together. Resolution in light microscopy is governed in part by the wavelength of the light used to illuminate the specimen (the shorter the wavelength the greater the resolution) (Prescott et al., 1998; Becker et al., 1999).
Modern light microscopy can produce images of specimens to about 2,000 X magnification. Greater magnification is possible, indeed a light microscope can be built to yield a final magnification of 10,000. However, due to the resolution limit imposed by the wavelength of visible light any two objects closer together than 0.24 μm would be observed as a single structure. Living specimens can be directly examined with the light microscope. To increase visibility, however, the specimen must be fixed and stained. This also can serve to accentuate specific morphological features and preserve the specimens for future study (Smith and Wood, 1996; Prescott et al., 1998).

The magnification and resolution of the electron microscope is much greater than that of the light microscope. In general, the electron microscope has a useful magnification power of 100,000 and a resolution limit of 2 nm. However, specimen preparation usually involves chemical fixation, dehydration, and staining or coating, and hence there is a risk of producing artifacts (Butt et al., 1990; Kasas et al., 1993). Living specimens cannot be examined with the electron microscope because of the vacuum to which specimens are exposed (Prescott et al., 1998; Becker et al., 1999).

The STM, in comparison, has greater magnification and resolution powers than the light and electron microscopes. It can achieve magnifications of 100,000,000 and has a resolution limit of 0.1 to 10 nm. When imaging using the STM a sharp tip is placed close enough to the surface of a conducting sample so the tunneling
of electrons between the two is made possible. The tunneling current provides an image that reflects the electronic structure and topography of the uppermost atoms at the surface of the sample. As STMs work on the basis of tunneling electrons between two conductive surfaces they are limited to imaging conductive specimens. Specimen preparation for STM examination, therefore, involves the adsorption of non-conductive specimens on to conductive substances such as specially treated graphite. The STM is therefore better suited to producing images of physical surfaces rather than biological specimens which are often good insulators (Smith and Wood, 1996; Prescott et al., 1998; Becker et al., 1999).

The AFM produces images of similar magnification and resolution as the STM, i.e. magnification of $10^6$ and resolution of 0.1 to 10 nm (i.e. Ångstrom resolution). The AFM has an advantage over the STM in that the sample does not need to be an electrical conductor. Therefore all types of specimen can be imaged using the AFM (Rugar and Hansma, 1990). Very little sample preparation is required when using the AFM compared to the electron microscope, thereby reducing the risk of artifacts and the AFM can be used in a fluid environment, providing the ability to examine living specimens (Becker et al., 1999).

In conclusion, use of the AFM offers greater magnification and resolution powers in combination with the least sample preparation and the ability to image both living and fixed specimens.
1.3.3: Biological applications of the AFM.

The invention of the AFM allowed the possibility of studying the surfaces of biological material at the molecular level. The AFM is now widely used to observe biomolecules, from whole cells down to smaller structures, such as membranes, proteins and nucleic acids. The breakthrough in biological applications of the AFM came with the development of the liquid cell, which permitted the observation of samples in their physiological environments. This allowed not only the observation of the structure of biological molecules but also the investigation of their dynamic processes.

Cells investigated using the AFM include erythrocytes, leukocytes, plant cells and cultured cells. Erythrocytes were imaged with the AFM in 1996 by Zachee et al. and further investigations have involved the imaging of *Plasmodium falciparum-*infected erythrocytes (Garcia et al., 1997). The imaging of leukocytes and plant cells has also been performed (Butt et al., 1990). The AFM has frequently been employed to observe both routine cultured cell lines and cultured cancerous human cells. Examples include chicken cardiocytes (Hofmann et al., 1997), CV1 kidney cells (Le Grimellec et al., 1998; Lesniewska et al., 1998), renal epithelium (Oberleither et al., 1996), MDCK cells (Lesniewska et al., 1998), cancer cells, fibroblasts and macrophages (Braet et al., 1998). The AFM has also been employed to study the surface dynamics of living acinar cells (Schneider et al., 1997) and to visualise the motility, division, aggregation, transformation and apoptosis of living cells (Kuznetsov et al., 1997). Vaccinia
virus-infected monkey kidney cells have also been observed using the AFM (Haberle et al., 1992).

Both lipid membranes (Egger et al., 1990; Weisenhorn et al., 1990) and membrane proteins (Butt et al., 1990; Hoh and Hansma, 1992; Yang et al., 1993) have been examined with the AFM. Individual proteins such as antibodies (San Paulo and Garcia, 2000), ferritin (Ohnishi et al., 1993), Escherichia coli RNA polymerase (Kasas et al., 1997) and Staphlococcus aureus heat shock protein (Ohta et al., 1996) have also been imaged using the AFM.

Nucleic acids such as DNA (Bustamante et al., 1992; Henderson, 1992; Yang and Shao, 1993; Lyubchenko et al., 1997) and RNA (Kalle et al., 1996) have been visualised using the AFM. Investigations such as length measurements of DNA (Hansma, 1997), binding of transcription factors to DNA (Nettikadan et al., 1996) and changes in the macromolecular conformation of DNA in vitro have also been performed.

The AFM has also been applied to virological and microbiological disciplines with the imaging of viruses (Larson et al., 1993; Bushell et al., 1995; Falvo et al., 1997; Maeda, 1997; Drygin et al., 1998; Keller et al., 1998; Malkin et al., 1999), bacteriophages (Kolbe et al., 1992) and halobacteria (Butt et al., 1990).
The AFM has been applied to investigate the dynamic processes of biological samples. In 2000, Jass et al. visualized the sequence of events involved in the transition from attached liposomes to bilayer patches on hydrophilic and hydrophobic surfaces. The process of pox virus infection of living cells has also been observed (Ohnesorge et al., 1997).

In 1991, Haberle et al. performed force microscopy on living cells. Since then, the application of the force spectroscopy capabilities of the AFM has lead to a new field of exploration of biological samples. The elasticity of normal and cancerous human bladder cells (Lekka et al., 1999), human fibroblasts (Bushell et al., 1999), MDCK cells (A-Hassan et al., 1998) and cartilage (Weisenhorn et al., 1993) have been investigated using force spectroscopy. Force spectroscopy has also been used to assess the forces exerted between biological molecules such as antibody-antigen and ligand-receptor interactions. It had been anticipated that the use of ligand-coated tips in combination with force measurements would allow receptors to be detected and localized on cell surfaces. Indeed, in 2000, Lehenkari et al. reported the mapping of calcitonin-calciitonin receptor binding forces in living bone cells. In the future it may be possible to develop a multi-antibody coated tip system which would allow the detection of specific antigens by detecting the attractive forces between the antibody and antigens.
Since its invention in 1986, the AFM has contributed hugely to the biological sciences. As more biologists become familiar with its use and endless capabilities, the quantity of scientific data provided by the AFM should increase exponentially.

1.3.4: AFM principle of operation.

The AFM uses a sharp tip mounted at the end of a flexible cantilever to probe a number of properties of the sample, including its topographical features and its mechanical characteristics. Precise lateral and vertical displacement of the probe with respect to the sample is achieved by a computer-controlled piezoceramic (a material which expands or condenses as a result of voltage applied), holding the cantilever. Forces acting between the surface and the probe cause deflection of the cantilever, this is registered by a laser beam reflected off the back of the cantilever, which is used to create a topographic image of the sample (Figure 1.9) (Zlatanova et al., 2000).

Cantilevers are manufactured from silicon nitride, have a length of 100-200 μm and are less than 1 μm thick. The spring constant of a cantilever is approximately 0.1 Nm\(^{-1}\), which is much less than the intermolecular vibration spring constant of the atoms in a specimen (1 Nm\(^{-1}\)). This enables the cantilever to sense exquisitely small forces exerted by individual sample atoms. A pyramidal stylus at the cantilever end provides a tip sufficiently sharp to achieve a lateral
Figure 1.9 AFM principle of operation. The AFM tip, which is mounted on a flexible cantilever, is scanned over the sample surface. A laser is reflected off the cantilever back onto a position-sensitive photoelectric diode. As the tip 'feels' the sample surface the cantilever is deflected and the position of the reflected laser beam on the photoelectric diode is altered. The AFM computer software interprets the change of laser position as a data point with x, y and z coordinates, which are used to obtain the AFM image. The AFM software also uses the data to run the feedback system, which serves to keep the force exerted by the tip on the sample constant. This is achieved by applying a +/- voltage to the piezoelectric scanner.
resolution often greater than 1 nm. The AFM tip is also constructed of silicon nitride and is 10-15 μm long. The geometry of the AFM tip is important with regard to the maximum achievable resolution in the x-y plane (Figure 1.10). When imaging extremely flat surfaces, this is determined by the diameter of the atom(s) at the probe tip. Thus, tip geometry is not critical in atomic resolution imaging of an atomically flat sample. However, when imaging larger surface features, tip geometry determines image resolution and generally, the sharper the tip, the better the resolution of the image (Lai and John, 1994; Marek, 1996a; Engel et al., 1999).

The back of the cantilever is a reflective surface. A laser beam is focused onto the back of the cantilever, this beam reflects off the cantilever and is directed onto a position-sensitive photoelectric diode. When the tip is not in contact the beam is positioned onto the center of the diode. The tip is advanced towards the sample until the two come into contact. A small loading force is applied and the x-y piezoelectric raster scans (Figure 1.11) the tip in the x-y plane over the sample. As the tip moves over the contours of the sample surface, the cantilever is deflected by the interatomic forces between the tip atoms and the sample atoms. The deflection of the cantilever alters the position of the reflected laser beam on the diode. This change in position is recorded by the software and converted into a visual image of the sample (Rugar and Hansma, 1990; Lal and John, 1994).
Figure 1.10 AFM tip geometry. The geometry of the AFM tip can falsely affect the topographical data of an image. A The tip has a 'blunt' geometry, hence the topographic data obtained shows a false curved feature instead of the true sample feature. B The AFM tip has a sharp pyramidal geometry and can accurately 'sense' the sample surface.
Figure 1.11 Raster scans. The diagram shows the direction of tip movement across a sample surface during the raster scan process. Movement of the tip and cantilever in the x-y planes is achieved via the piezoelectric scanner.
The change in position of the reflected laser beam on the diode also supplies the information to drive the feedback system. This system ensures that the force between the tip and the sample remains constant. It achieves this via the z piezoelectric scanner, which is attached to the tip. The scanner is fabricated from a material, which expands or condenses as a result of voltage applied. As the beam position on the diode changes, a voltage +/- is applied to the piezoelectric scanner, thus causing the scanner to condense or expand. This causes the tip to move in the z direction with respect to the sample and thereby maintains a constant force (Rugar and Hansma, 1990; Lal and John, 1994).

1.3.5: AFM imaging modes.

The AFM has a number of acquisition modes, each designed to obtain specific types of information from specific types of samples. The AFM can be operated in contact, non-contact, lateral force, modulated force, point spectroscopy and layered imaging modes (Wickramasinghe, 1990; Marek, 1996a).

In the contact mode of operation the probe tip scans across the sample surface, coming into direct physical contact with the sample and the AFM senses the repulsive forces between tip and sample. Contact AFM is the simplest AFM method, involving the least instrument variables for the gathering of topographic information. However, the force applied due to the direct contact between the tip and the sample can sometimes lead to deformation of soft samples (Marek, 1996a).
In the **non-contact mode** of AFM operation, the cantilever is oscillated at its resonant frequency above the sample surface. As the probe comes closer to the sample surface, the attractive force between the tip and the sample changes the oscillation amplitude and phase of the vibrating cantilever. The topographic data is produced by the feedback loop by detecting either the change in amplitude or phase. As there is no physical contact between the probe and the sample surface this mode is suitable for imaging soft or adhesive samples (Marek, 1996a).

The **lateral force mode** of operation is suited to accurately detecting the boundaries between different materials where the topographic change between the materials is minimal. In the lateral force mode the probe tip is scanned across the sample surface and the friction between the tip and sample causes the cantilever to flex laterally. This flexing can be detected and converted into an image (Marek, 1996a).

**Modulated force mode** maps variations in the hardness of a sample by detecting variations in the AC signal during contact imaging. This mode of imaging can be applied, for example, to mapping phase distributions of blended polymers where topographic data alone would not reveal the boundaries between the different polymer phases (Marek, 1996a).
In **point spectroscopy mode** the AFM is used to obtain a force-distance curve. This is achieved by positioning the probe at a point on the sample and pushing inward toward and/or outward from the surface. As the force increases, deflection is measured and a curve plotting deflection (force) versus distance is produced. Point spectroscopy can be used to reveal the relative softness or stiffness of any point of a sample (Marek, 1996a).

The **layered imaging mode** maps the sample surface to reveal magnetic, electrostatic, or compliance/adhesion patterns at varying levels of tip-sample spacing. Rather than scanning or dragging the probe tip across the sample surface each data point is gathered by creating a force-distance curve (Marek, 1996a).

**1.3.6: AFM computer software.**

The AFM computer software includes display, processing and analysis features. The image display software enables the enhancement of the visualization of the image. Images can be improved with regard to background subtraction, contrast, brightness, 3D display (Figure 1.12) and palette editing, without affecting the original data. The AFM processing software feature enables the adjustments of the image data with regard to curvature, leveling, convolutions, arithmetic processing and filtering (Marek, 1996b).
Figure 1.12 AFM contact mode images of BHK-21 cultured cells displayed by the AFM image analysis software. All parts of the figure reveal the same AFM image (the data has been adjusted with regard to leveling prior to display). A 100 μm x 100 μm 2D image display. B 100 μm x 100 μm 3D image display. C The roughness (Ra) analysis feature of the software. The Ra of the whole and a user-defined partial image are displayed. D The line analysis feature of the software. The positions of the user-defined line in the image, and the difference in height and distance between user-defined points in the line are displayed.
The image analysis software provides the tools to analyze the image with various statistical and measurement routines, such as line profile, peak and valley analysis, particle and grain analysis and step measurement. The line and roughness analysis features of the analysis software package were employed in this study (Figure 1.12). Line analysis enables the performance cross-sectional line analyses to determine the height and width of any feature within an image. The roughness analysis software enables the determination of roughness parameters for the entire image or a user-defined portion. The roughness (Ra) values referred to in this thesis represent the average roughness, which is the arithmetic mean of the absolute values of the measured profile height deviations (Marek, 1996b).
1.4: Objectives.

The subject of viral entry into cells and subsequent viral release has been extensively studied using numerous scientific techniques in fields ranging from cell culture to biochemistry to microscopy. One of the main structural differences which can exist between viruses, is the absence, or presence of a viral envelope and this structural factor has consequences for the mode of viral entry and release. The AFM is a novel developing microscopic instrument with unprecedented capabilities. The primary objective of this study was to observe enveloped (SFV) and non-enveloped (TMEV) viral entry into and egress from cultured cells using the AFM. The potential effects of anti-apoptotic factor Bcl₂ on viral entry and release was also investigated via the inclusion of the AT₃ cell line in the study. Virus particle structure of both enveloped and non-enveloped viruses was directly examined and the structure of viral capsids following the removal of the envelope was also investigated.

To achieve these objectives a number of techniques were developed and applied:

1. Optimisation of cell sample preparation for AFM examination.
2. Visualisation of enveloped and non-enveloped virus entry into and subsequent release from cultured cells.
3. Comparison of AFM-determined cultured cell viscoelasticity during enveloped and non-enveloped viral entry and egress.
4. Visualisation of enveloped and non-enveloped virus particles using the AFM.
Chapter 2

Materials and Experimental Methods
2.1: Materials.*

Chemicals were supplied by Sigma, Dublin, Ireland or BDH, Poole, England unless otherwise stated. Tissue culture media and serum were supplied by GibcoBRL, Paisley, Scotland. Tissue-culture plastic laboratory ware was supplied by Sarstedt, Drinagh, Co. Wexford, Ireland.

2.1.1: Cell culture.

Cell lines

BHK-21 cells were obtained courtesy of Dr. H. O'Shea (Department of Biological Sciences, CIT). The AT₃Neo and AT₃Bcl₂ cells were a gift from Dr. M. Scallan (Department of Microbiology, NUIC). The AT₃Neo and AT₃Bcl₂ cell lines were originally generated by transfecting AT₃ rat prostate carcinoma cells with pZIPNeo and pZIPbcl-2/neo, respectively (Levine et al., 1993).

Viruses

The TMEV strains BeAn, GDVII and DA were supplied by Dr. H. O'Shea (Department of Biological Sciences, CIT) as were the SFV strains L10 and M9. The A7 strain of SFV was a gift from Dr. M. Scallan (Department of Microbiology, NUIC).

*See appendix A for the more routine materials used in this study.
Dexamethasone (250 µM)

Dexamethasone was required as a supplement for the RPMI 1640 medium used when growing the AT₃ cell lines. 17.19 mg of dexamethasone was dissolved in 10 ml of sterile distilled water (sdH₂O), filter sterilised, aliquoted and stored at 4°C.

Poly-DL-Lysine

Utilised for cell adhesion. 10x stock solution: 50 mg of poly-DL-Lysine was dissolved in 5 ml of sdH₂O, filter sterilised, aliquoted and stored at -20°C. The stock solution was diluted in sdH₂O to 1x to obtain a working solution.

2.1.2: Terminal deoxynucleotid transferase (TdT) mediated UTP nick end-labelling (TUNEL).

DNase I buffer

30 mM Tris, 140 mM Sodium cacodylate, 4 mM MgCl₂, 0.1 mM Dithiothreitol.

The pH was adjusted to 7.2.

2.1.3: Immunocytochemistry.

Antibodies

The polyclonal rabbit anti-TMEV (60 mg/ml) was supplied by Dr. H. O’Shea (Department of Biological Sciences, CIT). The polyclonal rabbit anti-A5 (41 mg/ml) was a gift from Dr. M. Scallan (Department of Microbiology, NUIC).
Tris buffered saline (TBS)

137 mM NaCl, 5 mM TRIS. 4.4 ml of 1 M HCl was added per litre of buffer. Adjusted to pH 7.4. To obtain the TBS working solution Tween 20 was added to a final concentration of 0.1%.

2.1.4: AFM.

2.1.4.1: AFM fixatives.

2% paraformaldehyde-lysine-periodate (PLP)

Part A: Stock solutions

1. Phosphate buffer

80 mM Na₂HPO₄, 23 mM NaH₂PO₄, adjusted to pH 7.4

2. Lysine HCl

0.2M lysine HCl, adjusted to pH 7.4

Part B: Fixative

4 g of paraformaldehyde was added to 5 ml distilled water (dH₂O) and heated on a magnetic stirrer (N.B. do not exceed 70°C). While heating, 10 M NaOH was added dropwise slowly, until the solution clarified. The solution was removed from the heat and made up to 50 ml with dH₂O. 75 ml phosphate buffer, 75 ml lysine HCl and 0.9 g Na periodate was added. pH was approximately 7.4.
4% phosphate buffered formal saline

Formal saline was diluted to 4% in 1x PBS.

0.25% glutaraldehyde

1 ml glutaraldehyde (25% aqueous solution) was added to 99 ml 1x PBS.

4% paraformaldehyde

Part A: Stock solution 10% paraformaldehyde

10 g of paraformaldehyde powder was dissolved in 100 ml of dH2O and stirred for 24 hours (h). The solution was clarified with 1 N NaOH solution and filtered with Whatman paper prior to use.

Part B: Fixative 4% paraformaldehyde

Stock solution was diluted to 4% using 1x PBS.

Paraformaldehyde-Glutaraldehyde (PFG)

10 ml of 4% paraformaldehyde was added to 10 ml of 0.25% glutaraldehyde.

1% formaldehyde

1 ml of formaldehyde (37% solution) was added to 36 ml 1x PBS.

Ethanolamine

150 mM ethanolamine. Adjusted to pH 7.5
2.1.4.2: Virus particle analysis.

Low salt buffer (LSB)

0.15 M NaCl, 10 mM Tris. Adjusted to pH 7.4.

Sucrose solutions

The required amount of sucrose was dissolved in LSB e.g. to obtain a 30% sucrose solution 30 g of sucrose was dissolved in 100 ml of LSB.

Silicon wafers

Silicon wafers were supplied by Nanosensors (Wetzlar, Germany) and had the following characteristics: Dopant-B, resistivity 4-6 Ω/cm, orientation 111 and thickness 500 ± 20 μm.

Silicon wafer washing solution

0.7 g of potassium dichromate (K₂Cr₂O₇) was dissolved in 25 ml of dH₂O, placed on ice and 14.5 ml of sulphuric acid (H₂SO₄) was added.

Silanization solution

2% of n-Octadecyldimethylmethoxysilane (ODDMMS) dissolved in HPLC grade toluene.

Citrate acetate buffer

35 mM Citrate acid, 65 mM Sodium acetate. Adjusted to pH 5.
Phosphate buffered saline buffer (PBS)

150 mM NaCl, 100 mM NaH$_2$PO$_4$, 40 mM Na$_2$HPO$_4$. Adjusted to pH 7.4.

Phosphate buffered saline 0.1% Tween 20 (PBST)

150 mM NaCl, 100 mM NaH$_2$PO$_4$, 40 mM Na$_2$HPO$_4$, 0.1% Tween 20. Adjusted to pH 7.4.

Antibodies

The polyclonal rabbit anti-TMEV (60 mg/ml) was supplied by Dr. H. O'Shea (Department of Biological Sciences, CIT). The polyclonal rabbit anti-A5 (41 mg/ml) was a gift from Dr. M. Scallan (Department of Microbiology, NUIC). The monoclonal mouse anti-E1d (IgG1; 3.5 mg/ml) and monoclonal mouse anti-E2d (IgG2a; 6 mg/ml) were a gift from Dr. J. Fazakerley (Department of Veterinary Pathology, University of Edinburgh).

2.1.4.3: AFM instrumentation.

Images were obtained in contact or non-contact mode using the Explorer AFM from ThermoMicroscopes (Sunnyvale, CA., USA). Data acquisition and processing was performed on ThermoMicroscopes SPMlab software (version 4). Commercially microfabricated silicon contact Pointprobes™ cantilevers (Nanosensors, Wetzlar, Germany) and non-contact cantilevers (Ultrasharp, Moscow, Russia) were used in this study. Contact cantilevers had the following characteristics: length 441 μm; width 50 μm; thickness 10-15 μm; resonant
frequency 14-18 kHz; spring constant 0.18-0.45 N/m. Non-contact cantilevers had the following characteristics: length 90 μm; width 60 μm; thickness 2.4 μm, resonant frequency 470 kHz; spring constant 82.5 N/m.
2.2: Experimental Methods.

2.2.1: Cell culture.

2.2.1.1: Cell culture medium.

BHK-21 medium

BHK-21 cells were grown and maintained in Glasgow's minimal essential medium (GMEM) supplemented with 10% NCS, 10% TPB, 200 mM glutamine, 100 U penicillin/ml and 100 μg streptomycin/ml.

RPMI medium

AT₃ (AT₃Neo and AT₃Bcl2) cells were grown and maintained in RPMI 1640 medium, supplemented with 10% FCS, 60 U penicillin/ml, 60 μg streptomycin/ml, 1.1 μl/ml of dexamethasone and 500 μg/ml of G-418.

Plaque assay medium (PAM)

GMEM supplemented with 0.5% BSA, 200 mM glutamine, 100 U penicillin/ml, 100 μg streptomycin/ml, 75 μg DEAE-dextran/ml and 800 mg MgCl₂/ml.

2.2.1.2: Virus infection of cells.

The medium was decanted from two flasks (T175 cm²) of cells into a clean container. The monolayers were rinsed twice in 1x PBS. A vial of virus

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#See appendix B for the more routine cell culture experimental methods performed in this study.
suspension (approx. 1 ml) was added to one flask and the flask was rocked gently to spread the suspension evenly over the monolayer. The second flask was "mock-infected" with approximately 1 ml of 1x PBS to act as a control. Both flasks were incubated at room temperature for approximately one hour, each flask was rocked gently every 15 min. Approximately 20 ml of medium was added to each of the flasks which were then incubated at 37°C, 5% CO₂ for 2-3 days until approximately 50% cytopathic effect (CPE) was observed. Virus was harvested by pouring the medium from the infected flask of cells into a sterile 50 ml Falcon tube. The virus suspension was centrifuged at 2,500 g for 10 min. The supernatant was removed, aliquoted into sterile cryotubes and stored at -80°C. The virus suspension was sterility tested.

2.2.1.3: Neutralisation assay.

Part A - Setting up plates.

A flask of BHK-21 cells was examined for confluence using the inverted light microscope (TMS-F, Nikon, Japan). The cells were passed to obtain a cell suspension and a cell count was performed on the cell suspension. The amount of cell suspension required to set up 10 mm x 35 mm plates was calculated. Each plate was seeded with approximately 1 x 10⁶ cells, in 3 ml of BHK-21 medium and incubated 37°C, 5% CO₂. The cells were semi-confluent 24 h later and a neutralisation assay was then performed.
Part B - Neutralisation Assay.

Seven 1:10 serial dilutions of antibody were prepared in 1x PBS: to achieve this 12 μl of antibody was added to 108 μl of 1x PBS. Stock viruses were diluted in 1x PBS to $5 \times 10^2$ p.f.u./ml for SFV and $1 \times 10^3$ p.f.u./ml for TMEV. 108 μl of diluted virus was added to each antibody dilution and to a 108 μl 1x PBS virus control. The antibody:virus and virus control solutions were incubated at room temperature for 1.5 h.

5% Difco agar was melted and transferred to a 56°C water bath until required. The medium was decanted from the 10 mm x 35 mm plates into a clean container, care was taken not to splash any medium into the cells and the cells were rinsed twice with 1x PBS. 100 μl of the antibody:virus dilutions and virus control were added to a series of plates, in duplicate and allowed to absorb at room temperature for approximately 45 min. 2 control plates were "mock-infected" with 1x PBS. The plates were rocked gently every 15 min. The agar was cooled and diluted to 0.05% with warm PAM. Approximately 1.5 ml of agar medium was added to each plate and allowed to set at room temperature. Each plate was gently flooded with PAM and incubated at 37°C, 5% CO₂ for 2 to 3 days (until plaques were visible).

Part C - Fixation and staining.

The agar was decanted from the plates into a suitable container. The cells were fixed with formal saline for a minimum of 10 min. The formal saline was
decanted and the cells were stained with Toluidine Blue for approximately 10 min. The Toluidine Blue was decanted, the plates were rinsed with tap water, dried and the plaques were counted.

2.2.2: Terminal deoxynucleotide transferase (TDT) mediated UTP nick end-labeling (TUNEL).

Cells were seeded onto 4 well chamber slides (Lab-Tek, Nalge Nunc International, IL, USA) at a density of $2.5 \times 10^5$ cells/chamber for BHK-21 cells and $1.3 \times 10^4$ cells/chamber for AT$_3$ cells. The chambers were incubated at $37^\circ C$, 5% CO$_2$ overnight, then infected with virus, at a multiplicity of infection (MOI) of 5 and incubated at $37^\circ C$, 5% CO$_2$ until 25% CPE was attained. The cells were washed (NOTE: All washing steps were performed three times using TBS), and fixed with freshly prepared 4% paraformaldehyde solution for 30 min at room temperature. The cells were then washed, incubated in 0.3% Triton X-100 for 2 min and washed again. The positive reaction control cells were incubated with DNase I (Boehringer Mannheim, Germany) 5 µg/ml in DNase I buffer, in a humidified chamber for 30 min at $37^\circ C$. All other cells were incubated in TBS at room temperature. The TUNEL procedure was performed using a TUNEL kit (Boehringer Mannheim, Germany). The cells were washed twice with TBS, 50 µl of TUNEL reaction mixture was added to the positive control and test cells and 50 µl of label solution was added to the negative controls. The cells were then incubated in a humidified chamber for 60 min at $37^\circ C$. The cells washed, 50 µl of Converter-AP was added and the samples were incubated in a humidified
chamber for 30 min at $37^\circ$C. The cells were washed, 0.5 ml of substrate (NBT/BCIP) solution (Boehringer Mannheim, Germany) was added, the cells were incubated for 10 min at room temperature and subsequently washed.

2.2.3: Immunocytochemistry.

After completion of TUNEL, the cells were incubated for 30 min in 0.3% $\text{H}_2\text{O}_2$ in dH2O to remove endogenous peroxidase activity. They were then washed in TBS 3 x 4 min and subsequently the cells were incubated for 45 min in 20% goat serum to minimise non-specific binding. The goat serum was decanted and the cells were incubated in diluted primary antibody (anti-TMEV 1:300 dilution; anti-A5 1:400 dilution) for 1 h. The cells were then washed in TBS 3 x 5 min and diluted secondary antibody (1:100 dilution; Goat anti-rabbit (IgG), DAKO, Denmark) was added for 1 h. The cells were washed in TBS 3 x 5 min and subsequently incubated for 30 min with Vectastain universal elite ABC reagent (Vector Laboratories, CA, USA). The cells were then washed in TBS 3 x 5 min and incubated with DAB solution until the required intensity of staining was achieved (approx. 5 min). Finally the cells were washed twice in dH2O and mounted in aqueous mounting medium, prior to light microscopy examination.
2.2.4: AFM analysis.

2.2.4.1: AFM fixatives.

AFM imaging of BHK-21 cells.

An autoclave sterilised 25 mm x 25 mm coverslip was placed flat inside each 35 mm x 10 mm tissue culture dish used. The coverslip/dish was seeded with approximately $0.14 \times 10^8$ cells in 3 ml of BHK-21 medium. The cells were incubated overnight at $37^\circ C$, 5% CO$_2$. Cells were washed twice in 1x PBS and fixed with the appropriate fixative (see below). Subsequently the cells were washed twice in 1x PBS (except for the ethanol-acetic acid fixed samples), the coverslip was removed from the tissue culture dish and the cells air dried. Samples were then examined using a light microscope (YS2-T, Nikon, Japan) and an area suitable for AFM imaging was marked.

2% paraformaldehyde-lysine-periodate (PLP), 0.25% glutaraldehyde and paraformaldehyde-glutaraldehyde (PFG)

The dish was flooded with the appropriate fixative for 20 min at $0^\circ C$. The fixation was stopped by flooding the dish with ethanolamine for 20 min at $0^\circ C$.

4% phosphate-buffered formal saline, 1% formaldehyde, methanol-acetone and formal saline.

The dish was flooded with the appropriate fixative for 10 min at room temperature.
4% paraformaldehyde

The dish was flooded with 4% paraformaldehyde for 30 min at room temperature. The cells were rinsed 2 x 5 min in 1x PBS.

Ethanol-acetic acid

The dish was flooded with 3 parts ethanol to 1 part acetic acid for 20 min at room temperature. The ethanol-acetic acid was removed and the coverslip blotted lightly. The dish was flooded with 96% ethanol for 5 min at room temperature and the cells were subsequently air dried.

2.2.4.2: Sample preparation for viral entry studies.

An autoclave sterilised 25 mm x 25 mm coverslip was placed flat inside each 35 mm x 10 mm tissue culture dish used. The coverslip/dish was seeded with enough cells in 3 ml of medium to ensure semi-confluence at time of infection (approx. $5 \times 10^5$ BHK-21 cells and $1 \times 10^5$ AT$_3$ cells) and incubated overnight at $37^\circ C$, 5% CO$_2$. Cultured cells were infected with the selected virus strain at an MOI of 5. The infection proceeded for 1 h and the sample was fixed in 4% paraformaldehyde. Three separate areas of the sample were imaged with the AFM in contact mode.

2.2.4.3: Sample preparation for release of virus progeny studies.

An autoclave sterilised 25 mm x 25 mm coverslip was placed flat inside each 35 mm x 10 mm tissue culture dish used. The coverslip/dish was seeded
with enough cells in 3 ml of medium to ensure semi-confluence at the time point of 25% CPE (approx. $5 \times 10^5$ BHK-21 cells and $1 \times 10^5$ AT$_3$ cells) and incubated overnight at 37°C, 5% CO$_2$. Cultured cells were infected (see section 2.2.1.2) with the appropriate virus strain at an MOI of 5. After infection the sample was incubated (37°C, 5% CO$_2$) until the required level of CPE was attained. The sample was subsequently fixed in 4% paraformaldehyde and three separate areas of the sample were analysed using the AFM in contact mode.

2.2.5: Force Curves.

2.2.5.1: Sample preparation.

An autoclave sterilised 25 mm x 25 mm coverslip was placed flat inside each 35 mm x 10 mm tissue culture dish used. The coverslip/dish was seeded with enough cells in 3 ml of medium to ensure semi-confluence at the time of infection (approx. $5 \times 10^5$ BHK-21 cells and $1 \times 10^5$ AT$_3$ cells) and incubated overnight at 37°C, 5% CO$_2$. For force curve analysis of viral entry, cultured cells were infected with the appropriate virus strain at an MOI of 10, the infection proceeded for 1 h and the sample was fixed in 4% paraformaldehyde. For force curve analysis of the release of progeny virus cultured cells were infected at an MOI of 5. When the required level of CPE was attained the samples were fixed in 4% paraformaldehyde.
2.2.5.2: Performance of force curves.

Cells were imaged with the AFM in the layered imaging mode using a non-contact cantilever. After an image was obtained the system was left in feedback and force measurements were acquired via the AFM force measurement software. Force measurements were taken from three different locations on the cell surface and from a glass reference. For each force measurement (glass and cell) taken a distance versus deflection plot was produced which was exported to a spreadsheet (Microsoft Excel version 4). The distance values for the glass and cell measurements were calculated using linear regression analysis. Indentation values were then determined by calculating the distance differences between the glass and cell measurements. Loading force values were obtained from the deflection data using the following equation:

\[
\text{Force (nN)} = \frac{\text{cantilever spring constant}}{\text{sensor response}}
\]

The loading forces and corresponding indentation values were plotted on an xy graph, thereby producing a force versus indentation (F/I) plot (Figure 2.1). The slope of this plot, given by the equation of the line \(y=mx+c\) where \(m\) is the slope, represented the relative cell viscoelasticity. The three cell viscoelasticity values obtained from each cell were averaged by the standard error of the mean technique to yield a mean cell viscoelasticity value for each sample analysed.
**Figure 2.1** Sample force verses indentation (F/I) plot. The relative cell viscoelasticity was determined from the slope of this plot, given by the equation of the line \(y=mx+c\), where \(m\) is the slope. In this example the relative cell elasticity is approximately 108 nN/nm.
2.2.6: Virus Particle Analysis.

To perform virus particle analysis it was first necessary to purify the virus strains. BHK-21 cells were infected with the selected virus strain. Infected cells and supernatant were harvested when 70% CPE was reached for SFV and 50% CPE for TMEV. The virus suspension (supernatant) was then frozen at -70°C until purification. The appropriate purification procedure was performed (see sections 2.2.6.1 for SFV and 2.2.6.2 for TMEV). Post-purification the virus pellet was resuspended in 500 μl of LSB.

2.2.6.1: SFV purification.

The virus suspension was thawed and clarified by centrifugation at 7,000 rpm (4°C) in a Beckman (model J2-21) centrifuge for 20 min. The pellet was discarded and the virus was precipitated overnight at 4°C with 7% w/v PEG 8000 and 2.3% w/v NaCl. The precipitated virus was pelleted by centrifugation (as before) and resuspend in 1 ml of LSB. The virus suspension was loaded onto preformed 20 to 70% sucrose gradients prepared in clear SW41 tubes and banded by centrifugation for 1.5 h at 35,000 rpm in a Beckman ultracentrifuge at 4°C. The virus band (formed in the middle of the gradient) was harvested, diluted in LSB, and pelleted for 1 h at 35,000 rpm (4°C) in an SW41 rotor. The pelleted virus was resuspended in LSB.
2.2.6.2: TMEV purification.

The virus suspension was thawed and clarified by centrifugation at 7,000 rpm (4°C) in a Beckman (model J2-21) centrifuge for 20 min. The pellet was discarded, 10% w/v N-Lauroyl sarcosine and 0.05% v/v 2-mercaptoethanol was added to the supernatant. The virus suspension was loaded into a clear SW41 tube and underlayed with 4 ml of 30% sucrose. The virus was pelleted by centrifugation at 4°C for 4 h at 27,000 rpm in a Beckman ultracentrifuge. The supernatant and sucrose cushion were discarded. The pelleted virus was resuspended in LSB.

2.2.6.3: Preparation of silicon wafers.

The wafer was placed in washing solution and incubated at 120°C for 2 h, rinsed in 0.2 μm filtered dH₂O and subsequently in HPLC grade acetone. The wafer was then dried under a stream of N₂ gas, placed in the silanization solution for 30 min at room temperature and washed in HPLC grade acetone until it appeared highly polished. Subsequently the wafer was dried under a stream of N₂ gas.

2.2.6.4: Application of antibody monolayers.

Stock antibody solutions were diluted to 20 μg/ml in 0.1 M citrate acetate buffer, pH 5. Silanised wafers were covered with the appropriate antibody dilution, placed in a humidified chamber and incubated at 37°C for 2 h. Following
incubation, the wafers were washed thoroughly in PBST, rinsed in 0.2 \( \mu \text{m} \) filtered distilled H\(_2\)O and dried under N\(_2\) gas.

2.2.6.5: Application of purified virus sample.

To analyse SFV capsids, sodium dodecyl sulphate was added to purified L10 (enveloped) virus, to a final concentration of 2\% and the sample was incubated at 37\(^\circ\)C for 1 h. Purified virus stocks were diluted, when necessary, in PBS and pipetted onto the wafer surface. The concentration of virus applied was calculated via the area of the virus particle, the area of the wafer, the titre of the virus suspension and the volume of liquid that could be applied to the wafer surface (see appendix 3). The wafers were then incubated in a humidified chamber at room temperature for 1.5 h. Following incubation, the wafers were washed in PBST, rinsed in 0.2 \( \mu \text{m} \) filtered distilled H\(_2\)O and dried under N\(_2\) gas. Wafers were stored in a dessicator prior to imaging.
Chapter 3

Optimisation of cell sample preparation for AFM examination
In order to examine the entry and subsequent emergence of virus from cultured cells, the optimisation of cell preparation for atomic force microscopy (AFM) examination was investigated. This required the determination of three factors. Firstly, the matrix on which to culture the cells, secondly, the optimal concentration of cells to seed on this matrix and finally, the method by which to fix the cells. Other investigators have imaged cultured cells using a variety of matrices and fixatives (Haberle et al., 1992; Braet et al., 1997; Hofmann et al., 1997; Weyn et al., 1998). The methodology of each investigator was considered prior to optimisation of each factor.

3.1: Selection of sample matrix.

Other authors have reported that glass coverslips are the most frequently encountered matrix upon which to culture cells for AFM examination (Ricci and Grattarola, 1994; Braet et al., 1996; Haydon et al., 1996; Obelerleithner et al., 1996; Braet et al., 1997; Garcia et al., 1997; Kuznetsov et al., 1997; Schneider et al., 1997; Le Grimellec et al., 1998; Ushiki et al., 1999). Other matrices used include 35 mm X 10 mm petri dishes (Hofmann et al., 1997), microscope slides (Kasas et al., 1993; Zachee et al., 1996; Weyn et al., 1998) and patch clamp pipettes (Haberle et al., 1992; Horber et al., 1992). As the coverslips were previously the most widely used matrix they were selected as the matrix in this study. Sterility was required as a standard condition for cell culture equipment. Several papers cited coating of coverslips with adhesion factors such as poly-D-lysine (Ricci and Grattarola, 1994), collagen (Braet et
al., 1997), Cell-Tak (Schneider et al., 1997) and laminine (Ricci and Grattarola, 1994). Others, however, omitted this step (Braet et al., 1996; Haydon et al., 1996; Obelerleithner et al., 1996; Garcia et al., 1997; Kuznetsov et al., 1997; Le Grimellec et al., 1998; Ushiki et al., 1999). Data from these studies provided a basis for the development of the initial pilot experiments as follows. Glass coverslips (22 mm x 22 mm) were sterilised by autoclaving at 121°C for fifteen minutes. A single coverslip was placed in each 35 mm x 10 mm tissue culture dish. BHK-21 cells were seeded and maintained in culture medium (refer to section 2.2.1.1). The cells were incubated overnight at 37°C in 5% CO₂. Twenty-four hours later the cells had attained confluency and were examined using the AFM.

3.2: Selection of cell seeding density.

Initial images obtained indicated that when in a confluent state BHK-21 cells were unsuitable for AFM imaging. The vertical heights of the sample were resulting in saturation in the z direction of the AFM piezo. To compensate for this, seeding values which ensured sub-confluency at the fixation time were employed (Table 3.1). This problem was never encountered with the AT₃ cells (refer to section 2.1.1) as these do not naturally develop to a stage of monolayer confluence. It is assumed that cell lines, which reach monolayer confluency, such as fibroblastic cells, should always be examined in a sub-confluent state with the AFM. Ricci and Grattarola (1994), recommend the imaging of cells in a sub-confluent state when imaging 3T6 (fibroblastic) cells.
<table>
<thead>
<tr>
<th>Cell type</th>
<th>Seeding density$^*$</th>
<th>Cell density 24 hrs post seeding$^#$</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHK-21</td>
<td>$5 \times 10^5$</td>
<td>$7 \times 10^5$</td>
</tr>
<tr>
<td>AT$_3$Neo</td>
<td>$1 \times 10^5$</td>
<td>$4 \times 10^5$</td>
</tr>
<tr>
<td>AT$_3$Bcl$_2$</td>
<td>$1 \times 10^5$</td>
<td>$4 \times 10^5$</td>
</tr>
</tbody>
</table>

Table 3.1 Typical cell seeding densities. $^*$Seeding values given are those for a 22 mm x 22 mm coverslip in a 35 mm x 10 mm tissue culture dish. $^\#$Sub-confluent cell densities 24 hours (h) post-seeding represent cells suitable for imaging.
When imaging cell lines that do not attain monolayer confluence, i.e. AT₃ cells, it is not necessary to adhere to this requirement.

3.3: Selection of cell fixative.

Having determined the matrix on which to culture cells and the state of confluence at which they should be examined, the method of fixation was then addressed. Nine commonly-used cell fixation techniques were selected for the fixation trials. The fixatives chosen included formal saline, 4% phosphate-buffered formal saline, 1% formaldehyde, 0.25% glutaraldehyde, paraformaldehyde-glutaraldehyde (PFG), 4% paraformaldehyde, 2% paraformaldehyde-lysine-periodate (PLP), ethanol:acetic acid and methanol:acetone. For the fixation methods refer to section 2.2.4.1. The fixation trials were performed on BHK-21 cells. Duplicate trials were performed on each fixation technique. From the images obtained at this stage the three most promising fixatives (4% paraformaldehyde, 2% PLP and formal saline) were exposed to a third trial before a final selection was made.

To determine which fixative was to be selected, it was first necessary to define the selection criteria. As a general assumption, a fixative must preserve the cell in as "life-like" a state as possible without the generation of artifact. Artifact is a term used to indicate the presence of a property not normally associated with the cell. As regards to the former criterion, it is assumed that the fixatives selected for trial are capable of maintaining cells in
true morphological states, since the chosen reagents are all widely accepted cell fixatives. As the structure of BHK-21 cells has not previously been elucidated using the atomic force microscope, there was no data available on the cellular features that could be revealed by the AFM images. However, it is possible to extrapolate from light microscope images (observed prior to AFM imaging) of the cells, and obtain a general comparison of cell shape i.e. fibroblastic cell morphology.

Additional fixation criteria were also required to further reduce the possibility of artifact generation. These criteria can be tentatively divided into two categories, fixation artifact and imaging artifact (Table 3.2). Fixation artifacts include the presence of coatings, debris, crystals and depressions. Imaging artifacts, alternatively, include the occurrence of saturation and streaking. All of the above artifacts were encountered during the fixation selection process (Figures 3.1-3.4).

Coating artifact occurs essentially when the fixation process deposits a continuous layer of material, which dries over the sample surface. This coating interferes with the acquisition of cell surface topographic information with the AFM (Figure 3.1) and represents a limitation of the AFM in comparison to optical microscopy techniques. Coating can be detected by examining the roughness (Ra) parameters of the sample surface. The glass matrix should have a relatively low Ra value as it is a relatively smooth
<table>
<thead>
<tr>
<th>Artifact Type</th>
<th>Cause</th>
<th>Effect</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coating</td>
<td>Continuous layer of material covering sample surface.</td>
<td>Inaccurate Ra readings.</td>
<td>Alternative fixative used.</td>
</tr>
<tr>
<td>Debris</td>
<td>• Post-fixation cellular disintegration.</td>
<td>False topographic data.</td>
<td>• Alternative fixative used.</td>
</tr>
<tr>
<td></td>
<td>• Insufficient removal of PBS wash.</td>
<td></td>
<td>• Maximum removal of PBS.</td>
</tr>
<tr>
<td>Crystal</td>
<td>Salt crystals derived from PBS.</td>
<td>False topographic data.</td>
<td>Filtration of PBS.</td>
</tr>
<tr>
<td>Depression</td>
<td>Dehydration of cells.</td>
<td>False topographic data.</td>
<td>Alternative fixative used.</td>
</tr>
<tr>
<td>Imaging</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saturation</td>
<td>Cell height exceeding AFM z piezo capabilities.</td>
<td>False topographic data.</td>
<td>Alternative fixative used.</td>
</tr>
<tr>
<td>Streaking</td>
<td>Adherence of AFM tip to cells or loosely bound debris</td>
<td>False topographic data.</td>
<td>Alternative fixative used.</td>
</tr>
</tbody>
</table>

Table 3.2 Fixative and imaging artifacts. Listed above are the fixative and imaging artifacts encountered during experiments performed to select a fixative for cultured cell imaging.
Figure 3.1 Diagram illustrates the effect of coating artifact on acquisition of topographic data. A The AFM tip scans the cell surface and records 'true' topographic data. However, in B the presence of a coating prevents the tip from scanning the cell surface and data obtained represents 'false' topographic data.
surface. Alternatively, the cell surface should have a high Ra value, contributed by the natural undulations of the cell surface. Coating is indicated when the glass and cell surface Ra values are the same or when the cell has an unusually low Ra value.

The presence of debris in the sample can obscure cell surfaces leading to 'false' topographic data. Debris could result from one of two sources (Table 3.2). Firstly, it may be a result of cell disintegration, post-fixation. As each of the fixatives investigated are accepted as model fixatives the cells should not be subject to morphological deterioration. However, the fixatives selected are routinely used for optical microscopic techniques, which are unaffected by the presence of transparent debris on topographic data. A second potential source of debris arises from the possibility that after the final wash with phosphate-buffered saline (PBS), the liquid was not sufficiently removed prior to air-drying, thereby enabling any debris present to be dried onto the sample. This effect was investigated, and it was noted that when the maximum amount of fluid was removed from the sample, the presence of debris was significantly reduced.

Crystal artifact (Figure 3.2) was encountered frequently during the selection process. This artifact is not very obvious on the topographic images of the cells and was only seen on the glass matrix. It is possible, however, that the crystals were present on the cell surfaces but were obscured by the large cell
**Figure 3.2** Topographic image of crystal artifact detected on glass substrate surface.
surface height data. Therefore, it was important to reduce the occurrence of crystal artifact due to the possible presence of crystals on the cell surface. To achieve this it was necessary to discover the source of the crystals. A literature search revealed that Bushell et al. (1995) had previously encountered crystal artifacts and determined that the crystals were in fact salt crystals derived from the washing buffer (PBS). To remove these crystals the PBS was filtered with a 0.45 μm filter. The filtering of the fixative itself was also investigated, however, this resulted in reduced fixative capabilities. It was determined that filtered PBS used in each step of a fixation process was sufficient to eliminate the occurrence of this fixation artifact.

Saturation artifact (Figure 3.3) occurs when the height of the cells exceeds the height capabilities of the AFM. This feature is represented in the topographic image as a flat plateau, which occurs at the higher parts of the cell. Saturation artifact prevents detailed examination of the topography in the area in which it occurs and was encountered with two of the fixatives investigated in the study. BHK-21 cell height data was not available for comparison, thus it was unclear if the cell heights naturally exceeded the height range of the AFM used in the study. If it is assumed that the cell height naturally exceeds the AFM height range then those fixatives that do not result in saturation must be reducing the cell height. Conversely, if it is assumed that the cell height does not naturally exceed the AFM height range then when saturation occurs it is possible that the fixative is affecting the cell
Figure 3.3 Shaded image of the 1% formaldehyde fixed BHK-21 cell visualised in Figure 3.7. Note the flat plateau, which occurs when the cell's height data exceeds the height capacity of the AFM.
height and increasing it to saturation. In any event saturation must be avoided as it can only be counteracted by using an AFM instrument with a greater height (z) range.

Streaking artifact occurs when the tip does not follow the cell surface contour accurately and occurs when material adheres to and is removed by the tip (Braet et al., 1998). Streaking artifact may arise when too much force is applied to the tip, in effect pushing the tip into the sample surface and making it difficult for the tip to disengage from the sample. In addition, if the tip is raster scanned over the sample surface too rapidly, the feedback loop may not have enough time to adjust to the position of the new data point. These sources of streaking may be counteracted by correct AFM instrumentation set-up. Streaking artifact may also occur as a result of a fixative characteristic. The fixed sample may be exerting adhesive forces onto the tip thus preventing it from disengaging or the sample may contain loosely bound debris which adheres to the tip during imaging. In this study, the AFM instrumentation was optimised prior to imaging and therefore, any streaking artifact which occurred can be attributed to the fixation technique.

Depression artifact is a term given to anomalies on the cell surface which are not postulated to be a true reflection of the cell surface. As BHK-21 cells have not previously been imaged with the AFM it is possible that the anomalies are a natural feature of the cell surface. However, if all the images obtained
throughout the process of selecting a fixative are considered, there are two types of depression artifact that can occur. An example of the first depression artifact is illustrated in Figure 3.4. As can be seen from this 3D topographic image there are volcano-like depressions on the surface of the cells. These depressions which are similar to those encountered by Kalle et al. (1996) and Weyn et al. (1998) and are the result of cell dehydration. The second depression artifact has a "valleyed" appearance and is not as prominent as the first.

To determine which of the nine fixatives investigated in this study was the most promising a qualitative grading system was devised. This system worked on the basis of awarding points to the fixative method when it satisfied the fixation criteria i.e. the presence of fibroblastic morphology concurrent with the absence of fixation artifact. One point was awarded to a fixative for the absence of the fixation artifact e.g. coating, saturation, depression and streaking artifacts. One to four points were awarded depending on the degree of fibroblastic morphology achieved, a four point score indicating an accurate representation of fibroblastic morphology based on light microscopy images. One to two points were awarded depending on the amount of debris present, the absence of debris was awarded two points, the presence of a small degree of debris one point and finally the presence of a significant amount of debris resulted in no points awarded. The grading system is summarised in Table 3.3.
Figure 3.4 Three dimensional view of ethanol:acetic acid fixed BHK-21 cells. Note the volcano-like structures present in the cells, it is postulated that these depressions are due to dehydration of the cells by the fixative.
<table>
<thead>
<tr>
<th>Fixative</th>
<th>Debris (/2)</th>
<th>Streaking Artifact (/1)</th>
<th>Coating Artifact (/1)</th>
<th>Saturation Artifact (/1)</th>
<th>Depression Artifact (/1)</th>
<th>Fibroblastic morphology (4)</th>
<th>Grade (/10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formal Saline</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>4% Phosphate-buffered formal saline</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>1% Formaldehyde</td>
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<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>5</td>
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<tr>
<td>0.25% Glutaraldehyde</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>6</td>
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<tr>
<td>PFG</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>2% PLP</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>4%</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>Paraformaldehyde</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethanol:acetic acid</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Methanol:acetone</td>
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<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>3</td>
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Table 3.3 Fixative efficiency grading scheme of duplicate fixation trials. Fixatives were graded as follows: Debris - 2 points if absent, 1 point if small amount present, 0 points if significant amount present. Streaking, coating, saturation and depression artifacts - 1 point if absent, 0 points if present. Fibroblastic morphology - 4 points awarded in ascending order i.e. 4 points for perfect fibroblastic morphology. Maximum scores under each heading are given in parentheses.
3.3.1: Formal Saline.

As can be seen in Figure 3.5, formal saline fixed BHK-21 cells have a fibroblastic morphology with excellent correlation to optical microscopy images. No fixation artifacts were evident and only a small amount of debris was noted. Formal saline was awarded a grade of nine under the devised grading scheme (Table 3.3).

3.3.2: 4% Phosphate-buffered formal saline.

BHK-21 cells fixed with 4% phosphate-buffered formal saline do not appear to have fibroblastic morphologies (Figure 3.6). The images obtained were covered in debris and the streaking artifact has occurred in a number of positions. No coating, saturation or depression artifacts were noted. From the images obtained, 4% phosphate-buffered formal saline fixed cells were awarded a grade of four (Table 3.3).

3.3.3: 1% Formaldehyde.

1% formaldehyde fixed BHK-21 cells were awarded a grade of five (Table 3.3). The cells imaged were of fibroblastic morphology (Figure 3.7). No depression, streaking or coating artifact occurred. Debris was evident on the substrate surface and the saturation artifact was clearly visible on the cell surface.
Figure 3.5 Topographic image of formal saline fixed BHK-21 cells. The cells have a fibroblastic morphology with excellent correlation to optical microscopy images. No fixation artifacts were evident and only a small amount of debris was noted.
Figure 3.6 Topographic image of 4% phosphate-buffered formal saline fixed BHK-21 cells. The cells do not appear to have fibroblastic morphologies. The image is covered in debris and the streaking artifact has occurred in a number of positions. No coating, saturation or depression artifacts were noted.
Figure 3.7 Topographic image of 1% formaldehyde fixed BHK-21 cells. The cells are of fibroblastic morphology. No depression, streaking or coating artifact occurred. Debris is evident on the substrate surface and the saturation artifact is clearly visible on the cell surface.
3.3.4: 0.25% Glutaraldehyde.

Glutaraldehyde (0.25%) fixed BHK-21 cells exhibited a fibroblastic morphology, however debris was present on the cell surface (Figure 3.8). No fixative artifacts could be detected giving 0.25% glutaraldehyde a grade of six (Table 3.3).

3.3.5: Paraformaldehyde-glutaraldehyde (PFG).

BHK-21 cells fixed with PFG were awarded a grade of four (Table 3.3). As can be seen in Figure 3.9 the cells imaged do not have a fibroblastic morphology. Saturation artifact was evident and there was a significant amount of debris present. The debris appeared to have a regular shape and was present on the cellular surface. No coating, depression or streaking artifacts were noted.

3.3.6: 2% Paraformaldehyde-lysine-periodate (PLP).

The streaking artifact, shown in Figure 3.10, affected 2% PLP fixed BHK-21 cells. Debris was present on the substrate surface and also appeared to be on the cell surface. No coating, saturation or depression artifact were encountered and the cell is of fibroblastic morphology. The above factors lead to 2% PLP fixed BHK-21 cells being awarded a grade of seven (Table 3.3).
Figure 3.8 Topographic image of 0.25% glutaraldehyde fixed BHK-21 cells. The cells exhibited a fibroblastic morphology, however debris is present on the cell surface. No fixative artifacts could be detected.
Figure 3.9 Topographic image of PFG fixed BHK-21 cells. The cells do not have a fibroblastic morphology. Saturation artifact is evident and there is a significant amount of debris present. The debris appears to have a regular shape and is present on the cellular surface. No coating, depression or streaking artifacts are noted.
Figure 3.10 Topographic image of 2% PLP fixed BHK-21 cells. Streaking artifact is evident and debris is present on the substrate surface and also appears to be on the cell surface. No coating, saturation or depression artifacts are evident and the cell is of fibroblastic morphology.
3.3.7: 4% Paraformaldehyde.

BHK-21 cells fixed in 4% paraformaldehyde were awarded a grade of ten (Table 3.3). As shown in Figure 3.11 no depression, coating, saturation or streaking artifacts occurred. There was no debris evident from the images obtained and fibroblastic cellular morphology was conserved.

3.3.8: Ethanol:acetic acid.

Artifacts affected images of BHK-21 cells fixed with ethanol:acetic acid (Figure 3.12). The coating artifact affected images from the first attempt at fixing cells with this fixative. The images from the second trial revealed volcanic depression artifact. These depressions are similar to those encountered by Kalle et al. (1996) when investigating the influence of ethanol dehydration on cellular structures and are consistent with nuclear areas. No debris, saturation or streaking artifacts were encountered in either trial, thus the ethanol:acetic acid fixative was awarded a grade of six (Table 3.3).

3.3.9: Methanol:acetone.

BHK-21 cells fixed in methanol:acetone were awarded a grade of six (Table 3.3). Debris was present and "valleyed" depression artifact was also evident. A small amount of streaking artifact was present. No coating or saturation artifacts were encountered (Figure 3.13).
Figure 3.11 Topographic image of 4% paraformaldehyde fixed BHK-21 cells. No depression, coating, saturation and streaking artifacts occurred. There is no debris evident and fibroblastic cellular morphology was conserved.
Figure 3.12 Topographic image of ethanol: acetic acid fixed BHK-21 cells. The image reveals volcanic depression artifact. These depressions are similar to those encountered by Kalle et al. (1996) when investigating the influence of ethanol dehydration on cellular structures and are consistent with nuclear areas. No debris, saturation or streaking artifacts are evident.
Figure 3.13 Topographic image of methanol:acetone fixed BHK-21 cells. Debris is present and "valleyed" depression artifact is also evident. A small amount of streaking artifact is evident. No coating or saturation artifacts are revealed.
3.3.10: Final selection.

From the first two trials it was noted that the three most promising fixatives identified based on the qualitative grading system were 4% paraformaldehyde (grade 10), formal saline (grade 9) and 2% PLP (grade 7). All three fixatives were subjected to a third trial before a final decision was made. Images obtained of BHK-21 cells fixed in 2% PLP and 4% paraformaldehyde were consistent to those in the previous trials. Formal saline, however, did not give a consistent result. On the basis of this 4% paraformaldehyde was selected as the BHK-21 cell fixative of choice and was used as the cell fixative in the rest of this study.
Chapter 4

Analysis of the entry into and subsequent release of progeny virus from cultured cells
Atomic force microscopy (AFM) analysis of the entry into and subsequent release of virus from cultured cells is presented in this chapter. Additional techniques including cytopathic effect (CPE) studies, immunocytochemistry and terminal deoxynucleotidyl transferase (TdT) mediated UTP nick end labeling (TUNEL) were carried out to determine the optimal times to perform the AFM analysis and also to assist in the interpretation of AFM data. The chapter is divided into two sections; the first presents the examination of viral entry and the latter the subsequent emergence of progeny virus.

4.1: Analysis of viral entry.

4.1.1: CPE analysis.

To examine viral entry using the AFM it was first necessary to prove that the virus strain infected the cultured cell. To achieve this, CPE studies were performed on the cultured cells (BHK-21, AT₃Neo and AT₃Bcl₂), infected with the SFV (L10, M9 and A7) and TMEV (GDVII, DA and BeAn) virus strains. The CPE studies were performed in duplicate and repeated on three separate occasions. The cells were infected at a multiplicity of infection (MOI) of 5 and examined visually, using an inverted microscope, regularly until either complete CPE occurred or the uninfected control monolayer deteriorated. The appearance of the cells and the cell monolayer as a whole were examined and the degree of CPE present was assessed. The results of the CPE studies are presented in Tables 4.1 to 4.4.
CPE studies of BHK-21 cell infection with the viral strains are presented in Table 4.1. The cells were observed at a number of time points post-infection (p.i.) ranging from eight hours to seventy-five hours p.i. Of the TMEV strains it can be seen that the virulent GDVII strain produced a more rapid CPE (i.e. CPE changes were observed earlier and complete CPE was observed more rapidly) when compared to that which occurred with the avirulent DA and BeAn strains. The BeAn strain proved to have slightly more rapid CPE when compared to the DA strain. The SFV strains behaved in a similar manner to the TMEV strains. The virulent L10 strain induced a more rapid CPE when compared to the avirulent A7 and M9 strains. The A7 strain was narrowly more efficient than the M9 strain at producing the initial CPE, however both infections proceeded to develop complete CPE at the same time p.i. Overall, it was observed that all the virus strains examined do induce CPE in BHK-21 cells. Therefore, it can be assumed that GDVII, DA, BeAn, L10, M9 and A7 gain entry to BHK-21 cells and that this entry can be examined using the AFM. Figure 4.1 displays photographs taken, at a magnification of 200, of BHK-21 cells infected with the virus strains twenty-four hours p.i.

The results of CPE studies performed on AT₃Neo cells are presented in Table 4.2. Again, the cells were observed at frequent intervals ranging in this case from seven to seventy-six hours p.i. The TMEV strains failed to produce CPE in the AT₃Neo cell line, therefore it is likely that these cells do not support viral replication. The SFV strains however did successfully enter the cell line as CPE
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Table 4.1 Cytopathic effect of TMEV and SFV on BHK-21 cells. All samples were infected with a MOI of 5. Results were graded as follows; 0 = 0% CPE, (+) = slight CPE, + = 25% CPE, ++ = 50% CPE, +++ = 75% CPE and ++++ = 100% CPE.
Figure 4.1 BHK-21 cells infected with TMEV and SFV at an MOI of 5, 24 hrs p.i. (magnification 200). A Mock-infected control. B GDVII infected, 75% CPE. C L10 infected, 50% CPE. D DA infected, slight CPE. E A7 infected, 25% CPE. F BeAn infected, 25% CPE. G M9 infected, 50% CPE.
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**Table 4.2** Cytopathic effect of TMEV and SFV on AT₃Neo cells. All samples were infected with a MOI of 5. Results were graded as follows; 0 = 0% CPE, (+) = slight CPE, + = 25% CPE, ++ = 50% CPE, +++ = 75% CPE and ++++ = 100% CPE.
was observed. The initial CPE was visualized twenty hours p.i. and proceeded gradually until complete CPE was achieved. In contrast to the infection of BHK-21 cells with the SFV strains, the virulent L10 did not achieve a rapid CPE in comparison to the avirulent M9 and A7 strains. These results indicate that the L10 strain is either more proficient at gaining entry into or replicating in BHK-21 cells than in AT\textsubscript{3}Neo cells.

The CPE of TMEV and SFV on infected AT\textsubscript{3}Bcl\textsubscript{2} cells is shown in Table 4.3. The infected cells were observed at various time points beginning at seven hours and ending at seventy-six hours p.i. As with the AT\textsubscript{3}Neo cell line, infection with the TMEV strains did not produce CPE in the AT\textsubscript{3}Bcl\textsubscript{2} cells. CPE was observed when AT\textsubscript{3}Bcl\textsubscript{2} cells were infected with the SFV strains. The L10 strain produced a more rapid CPE than the A7 and M9 strains. The A7 strain induced a more rapid CPE in comparison to the M9 strain. Visualization of complete CPE induced by the M9 strain was not achieved as the experiment was terminated due to deterioration of the mock-infected control cells after the seventy-six hour p.i. time point.

The inability of TMEV strains to produce CPE in the AT\textsubscript{3}Neo and AT\textsubscript{3}Bcl\textsubscript{2} cell lines may be explained by either an inability of the virus to enter these cell lines or an inability, once entry has been achieved, of the virus to replicate within the cell. It was anticipated that AFM analysis of AT\textsubscript{3}Neo and AT\textsubscript{3}Bcl\textsubscript{2} cell lines infected with TMEV strains would elucidate this issue. A comparison of CPE induced by the SFV strains in AT\textsubscript{3}Neo and AT\textsubscript{3}Bcl\textsubscript{2} cells is presented in Table 114.
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**Table 4.3** Cytopathic effect of TMEV and SFV on AT₃Bcl₂ cells. All samples were infected with a MOI of 5. Results were graded as follows; 0 = 0% CPE, (+) = slight CPE, + = 25% CPE, ++ = 50% CPE, +++ = 75% CPE and ++++ = 100% CPE.
4.4. It is evident, for each of the SFV strains, that the onset of CPE is delayed in the AT₃Bcl₂ cells in comparison to the AT₃Neo cells. This can be attributed to the presence of the Bcl₂ transgene (which functions to delay apoptosis) in the AT₃Bcl₂ cell line and its absence in the AT₃Neo cell line (Levine et al., 1993). This effect is also illustrated in Figure 4.2 which displays photographs (magnification of 200) taken of AT₃Neo and AT₃Bcl₂ cells forty-five hours p.i. with the SFV strains.

4.1.2: Atomic force microscopy analysis.

AFM analysis to visualize the entry of virus into cultured cells was performed following the completion of the CPE studies. The entry of TMEV (GDVII, DA and BeAn strains) and SFV (L10, M9 and A7 strains) was analyzed to endeavor to elucidate any differences that might occur between enveloped and non-enveloped viruses at the stage of viral entry. Also, it was anticipated that the inability of the TMEV strains, as determined by the CPE studies, to induce CPE in the AT₃Neo and AT₃Bcl₂ cell lines would be more fully investigated using this approach.

To perform AFM analysis the cells were infected with the virus strains under the same conditions as those used in the CPE studies. The cells were fixed at the end of the infection time (one hour) in 4% paraformaldehyde and were subsequently analysed with the AFM in contact mode. The experiment was performed in duplicate for TMEV (GDVII, DA and BeAn strains) and SFV (L10
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**Table 4.4** Comparison of CPE induced by SFV strains in AT<sub>3</sub>Neo and AT<sub>3</sub>Bcl<sub>2</sub> cells. All samples were infected with a MOI of 5. Results were graded as follows; 0 = 0% CPE, (+) = slight CPE, + = 25% CPE, ++ = 50% CPE, +++ = 75% CPE and ++++ = 100% CPE.
Figure 4.2 AT₃ cells infected with SFV at an MOI of 5, 45 hours p.i. (magnification 200). A Mock-infected AT₃Neo cells. B Mock-infected AT₃Bcl₂ cells. C L10 infected AT₃Neo cells. D L10 infected AT₃Bcl₂ cells. E A7 infected AT₃Neo cells. F A7 infected AT₃Bcl₂ cells. G M9 infected AT₃Neo cells. H M9 infected AT₃Bcl₂ cells.
and M9 strains) infection of all three cell lines. SFV A7 strain infection was performed in duplicate in BHK-21 cells and once in AT₃Neo cells. For each sample analysed using the AFM, three separate areas of the sample were imaged, to ensure all possible features were observed.

The images obtained were examined for cell surface changes topographically and by using the line and roughness (Ra) analysis functions of the ThermoMicroscopes image analysis software. Prior to examination, the images obtained were levelled in the first order two-dimensional plane, and the data histogram was adjusted to limit the colour palette's distribution to the z-range of interest.

The Ra analysis function of the image analysis software provides an average roughness value in nanometers (nm). This represents the arithmetic mean of the deviations in height from the image as a whole or from a user-selected partial image. To analyse the Ra of the infected cells, three user-selected areas from each image of the infected cells, and their respective mock-infected controls were obtained. A mean Ra value for each control and virus infected cell line was then calculated. The Ra data obtained for each experiment in this chapter is illustrated graphically (Figures 4.3, 4.10, 4.12, 4.14, 4.18, 4.24 and 4.26).

As can be seen from Figure 4.3 the AT₃ cell lines have higher Ra values than the BHK-21 cell line. This indicates that the surface of BHK-21 cells is smoother than
Figure 4.3 Graph of roughness (Ra) from a user-defined area of infected and mock-infected cultured cells. BHK-21, AT3Neo and AT3Bcl2 cell lines were infected with the appropriate viruses at an MOI of 5. The cells were fixed in 4% paraformaldehyde one hour p.i. and subsequently imaged in contact mode with the AFM. Note that the AT3 cells have higher Ra values than the BHK-21 cells.
that of the AT₃ cell lines. No consistent increase or decrease in Ra values is observed for virally infected BHK-21 and AT₃Bcl₂ cells and no conclusions can be drawn from these results. However infected AT₃Neo cells have decreased Ra values when compared to the mock-infected control cells. This indicates that the surface of AT₃Neo cells becomes smoother during viral entry. If this smoothing effect is due to viral infection, it indicates that TMEV are capable of gaining entry into AT₃Neo cells and that CPE is not produced in these cells due to an inability of the viruses to replicate. It would be possible to conclude that viral infection causes the smoothing of infected cells if all three cell lines had shown a consistent result. However, this did not occur therefore it is impossible to categorically come to this conclusion until further studies are carried out.

Topographic and line analysis of the infected cells was performed and representative results are illustrated in Figures 4.4 to 4.9. The line analysis was performed by selecting a user-defined line across the image. No topographic and line analysis differences between the virus-infected cells and their respective mock infected controls were seen in the majority of cases. However, the cell heights of BHK-21 cells infected with GDVII (Figure 4.4) and A7 (Figure 4.5) were higher than those of the corresponding mock-infected control cells. This effect also occurred with GDVII (Figure 4.6), BeAn (Figure 4.6) and L10 (Figure 4.7) infected AT₃Neo cells and with DA (Figure 4.8) infected AT₃Bcl₂ cells. Payne and Kirstenson (1982) and Blasco and Moss (1991 and 1992) have reported that many virions remain adsorbed to the cell surface and never enter the interior of
Figure 4.4 Topographic and line analysis of mock-infected and TMEV infected BHK-21 cells. The cells were infected at a MOI of 5 and fixed one hour p.i. A= Mock-infected control, B-D= Cells infected with GDVII, DA and BeAn respectively.
Figure 4.5 Topographic and line analysis of mock-infected and SFV infected BHK-21 cells. The cells were infected at a MOI of 5 and fixed one hour p.i. A= Mock-infected control, B-D= Cells infected with L10, M9 and A7 respectively.
Figure 4.6 Topographic and line analysis of mock-infected and TMEV infected AT₃Neo cells. The cells were infected at a MOI of 5 and fixed one hour p.i. A= Mock-infected control, B-D= Cells infected with GDVII, DA and BeAn respectively.
Figure 4.7 Topographic and line analysis of mock-infected and SFV infected AT₃Neo cells. The cells were infected at a MOI of 5 and fixed one hour p.i. A= Mock-infected control, B-D= Cells infected with L10, M9 and A7 respectively.
Figure 4.8 Topographic and line analysis of mock-infected and TMEV infected AT₃Bcl₂ cells. The cells were infected at a MOI of 5 and fixed one hour p.i. A= Mock-infected control, B-D= Cells infected with GDVII, DA and BeAn respectively.
Figure 4.9 Topographic and line analysis of mock-infected and SFV infected AT3Bcl2 cells. The cells were infected at a MOI of 5 and fixed one hour p.i. A=Mock-infected control, B-C= Cells infected with L10 and M9 respectively.
the cell (Ohnesorge *et al.*, 1997). It is possible that the increase in cell heights reported above are due to aggregates of these adsorbed virions.

The possibility of imaging the samples too early in the infection process and thereby missing any conclusive cell changes was considered. To address this issue, a number of further samples were examined. BHK-21 cells infected with GDVII at a MOI of five were fixed two, four, five and six and a half hours p.i. After AFM imaging Ra, topographic and line analysis studies were performed. The results of the Ra analysis are illustrated in Figure 4.10. The graph shows that the Ra values of the infected cells are less than those of the mock-infected control. Of the infected samples the Ra is lowest at two hours p.i. after which time the Ra values rise but not consistently. Combining the Ra values from this experiment to that of the initial one hour p.i. investigation it is indicated that the Ra values of GDVII infected cells increases one hour p.i. but subsequently decreases two hours p.i. after which time it again increases. This may indicate using Ra values alone that GDVII starts to penetrate cells two hours p.i. However, when the corresponding topographic and line analysis data is examined (Figure 4.11), no appreciable difference between the control and infected cells is observed. To address this issue and try to obtain unconflicting evidence of viral entry, BHK-21 cells were infected with GDVII this time at a MOI of ten and fixed one, two, three and four hours p.i. Ra values for this experiment are illustrated in Figure 4.12. The graph in this case contradicts the previous Ra results in that in each hour p.i. the cells have higher Ra values than the mock-infected control. The topographic
Figure 4.10 Graph of roughness (Ra), from a user-defined area of infected and mock-infected cultured cells. BHK-21 cells infected with GDVII virus (MOI of 5) and fixed at 2, 4, 5 and 6.5 hours p.i. The Ra values of the infected cells are less than those of the uninfected control with the sample fixed 2 hours p.i. having the lowest Ra value.
Figure 4.11 Topographic and line analysis of mock-infected and GDVII infected BHK-21 cells. The cells were infected at a MOI of 5 and fixed at various time points p.i. A= Mock-infected control, B-E= 2, 4, 5 and 6.5 hours p.i. respectively.
Figure 4.11 continued
Figure 4.12 Graph of roughness (Ra), from a user-defined area of infected and mock-infected cultured cells. BHK-21 cells infected with GDVII virus (MOI of 10) and fixed at 1, 2, 3 and 4 hours p.i. All the infected cells have higher Ra values than the mock-infected control.
and line analysis result (Figure 4.13) did not reveal any changes between the mock-infected control and the infected cells.

In the event that entry of non-enveloped (TMEV) viruses into cultured cells could not be detected using the AFM an experiment was also performed using an enveloped (SFV) virus. BHK-21 cells were infected with the L10 strain at a MOI of ten and fixed at various time-points both during and after the viral infection procedure. The fixation times were fifteen minutes, thirty minutes, forty-five minutes, one hour, two hours, three hours and four hours. The combined Ra (Figure 4.14), topographic and line (Figure 4.15) analysis results from this study may represent the complete virus infection cycle. From CPE studies presented earlier in this chapter (Table 4.1), initial CPE was observed eight hours p.i. when BHK-21 cells were infected with the L10 virus strain at a MOI of five. In this experiment the BHK-21 cells were infected with L10 at a MOI of ten, and therefore initial CPE would have commenced much earlier than eight hours p.i. and significant CPE may have been achieved by four hours p.i. Adsorbed virus may be causing the increased Ra and slightly increased height values observed fifteen minutes into the infection process. Thirty minutes into the infection process Ra values are decreased and height values increased, these results may represent penetration of the cells by the virus. Fifteen minutes later Ra values have returned to normal levels, however the heights of the infected cells remain higher than the mock-infected control. Infected cell heights remain similar to the mock-infected control one and two hours p.i., while Ra values were raised.
Figure 4.13 Topographic and line analysis of mock-infected and GDVII infected BHK-21 cells. The cells were infected at a MOI of 10 and fixed at various time points p.i. A= Mock-infected control, B-E= 1, 2, 3 and 4 hours p.i. respectively.
Figure 4.13 continued
Figure 4.14 Graph of roughness (Ra), from a user-defined area of infected and mock-infected cultured cells. BHK-21 cells infected with L10 virus (MOI of 10) and fixed both during (15, 30 and 45 minutes) and after (1, 2, 3 and 4 hours) the infection procedure.
Figure 4.15 Topographic and line analysis of mock-infected and L10 infected BHK-21 cells. The cells were infected at a MOI of 10. A= Mock-infected control, B-D= Fixation times during the infection process, 15, 30 and 45 minutes respectively. E-H= Fixation times post-infection, 1, 2, 3 and 4 hours respectively.
Figure 4.15 continued
Figure 4.15 continued
and decreased respectively. Ra values were slightly increased three hours p.i. as were the infected cell heights. Four hours p.i., the cell heights have returned to levels similar to the mock-infected control. The Ra values however were significantly increased, indicating that the infected cell surfaces were rougher than the control possibly due to destruction of the cell membrane as the enveloped progeny viruses emerge. Also structures were evident on the cell surfaces post-infection (Figure 4.15, Parts E-H). These structures may represent aggregates of emerging virus or cellular blebbing, which occurs during apoptosis.

4.1.3: Summary of the analysis of viral entry.

One case in which the Ra values were indicative of viral entry effects on the cells was observed when AT3Neo cells were virally infected. In this case the TMEV and SFV infected AT3Neo cells were smoother than the mock-infected control. This result may indicate that TMEV are capable of gaining entry into AT3Neo cells and that CPE is not produced by TMEV in these cells due to an inability of the viruses to replicate.

The investigation of viral entry performed on BHK-21 cells infected with the L10 virus at a MOI of ten revealed some interesting results and may represent cellular changes during the complete viral infection cycle.

However, few conclusions can be drawn from the AFM analysis of viral entry into cultured cells in this study. The Ra, topographic and line analysis are indicative,
in some cases, of viral effects on the cell however as these are extremely variable from sample to sample it would be unwise to draw any conclusions from these results.

4.2: Analysis of the emergence of progeny virus.

The time p.i. of 25% CPE was chosen to attempt to visualize the emergence of progeny virus. To ensure that the virus was actively producing progeny virus at this time immunocytochemistry and TUNEL studies were performed.

4.2.1: TUNEL and immunocytochemistry analysis of infected cells.

Immunocytochemistry and TUNEL studies, on BHK-21, AT₃Neo and AT₃Bcl₂ cells infected with the TMEV and SFV strains, were performed in triplicate to ensure reproducibility of the results. As the TMEV infected AT₃Neo and AT₃Bcl₂ cell lines did not achieve 25% CPE the studies were performed sixty hours p.i. which represents the maximum time post-infection before mock-infected control deterioration. SFV infected AT₃Bcl₂ cells were assayed at two time points. The first was the time point at which the corresponding AT₃Neo cells achieved 25% CPE. The second time point was the time p.i. at which the virus induced 25% CPE in AT₃Bcl₂ cells.

Brown staining in the cytoplasm of the cell indicates a positive immunocytochemical reaction. The staining is achieved by an antibody-antigen
reaction between a viral protein (antigen) and an antibody specific for the protein. In the case of the SFV strains an anti-A5 antibody was used and anti-TMEV was employed to detect TMEV proteins. When a reaction occurs between the viral protein and its corresponding antibody it is detected by coupling the complex to a colour detection system with a brown-coloured end product. Blue/indigo staining in the nucleus of the cell indicates a positive TUNEL reaction and therefore the presence of apoptosis. This is achieved by labeling the DNA fragmentation which is characteristic of apoptotic cell death (Scallan et al., 1997). The results of the immunocytochemical and TUNEL studies are presented in Table 4.5 and Figures 4.16-4.17.

TMEV infected BHK-21 cells were positive for both the immunocytochemical and TUNEL studies. Immunocytochemical staining in the cell cytoplasm and TUNEL staining in the cell nucleus can be seen in Figure 4.16 parts D, G and J, the absence of staining is demonstrated in Figure 4.16 part A which represents mock-infected BHK-21 control cells. The CPE studies (Tables 4.2 and 4.3) indicated that TMEV strains are unable to produce CPE in the AT₃Neo and AT₃Bcl₂ cell lines. This was confirmed with the immunocytochemical and TUNEL studies. No immunocytochemical or TUNEL staining was achieved for GDVII, DA and BeAn infection of the AT₃Neo and AT₃Bcl₂ cell lines. This is illustrated in Figure 4.16 parts E, F, H, I, K and L. From the CPE, TUNEL and immunocytochemistry results it is evident that no virus progeny are produced following AT₃Neo and AT₃Bcl₂ cell infection with the TMEV stains. Therefore,
<table>
<thead>
<tr>
<th>Cell line</th>
<th>TMEV strains</th>
<th>SFV strains</th>
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<tr>
<td></td>
<td>GDVII</td>
<td>DA</td>
</tr>
<tr>
<td>BHK-21</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AT₃Neo</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AT₃Bcl₂⁺</td>
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<td>-</td>
</tr>
<tr>
<td>AT₃Bcl₂⁻</td>
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</tr>
</tbody>
</table>

**Table 4.5** Results of TUNEL and immunocytochemistry experiments. Cells were infected with a MOI of 5 and subsequently fixed with 4% paraformaldehyde. # Indicates that the AT₃Bcl₂ cells were fixed at the p.i. time corresponding to 25% CPE in AT₃Neo cells. * Indicates that the AT₃Bcl₂ cells were fixed at the p.i. time corresponding to 25% CPE in AT₃Bcl₂ cells. Table key is as follows; + indicates that the cells were positive for both TUNEL and immunocytochemistry, - indicates that the cells were negative for both TUNEL and immunocytochemistry and N/A indicates not applicable.
Figure 4.16 TUNEL and immunocytochemistry analysis of TMEV infected cells at 25% CPE. All cells were infected at an MOI of 5. A-C Mock-infected BHK-21, AT₃Neo and AT₃Bcl₂ cells respectively. D-F GDVII infected BHK-21, AT₃Neo and AT₃Bcl₂ cells respectively. G-I DA infected BHK-21, AT₃Neo and AT₃Bcl₂ cells respectively. J-L BeAn infected BHK-21, AT₃Neo and AT₃Bcl₂ cells respectively. NOTE: The presence of brown staining in the cytoplasm, indicating positive immunocytochemistry, and the presence of blue/indigo staining in the nuclei, indicating a positive TUNEL reaction, in D, G and J.
AFM analysis of the emergence of progeny virus on these samples was not performed.

CPE studies revealed that SFV strains have the ability to gain entry to and replicate in BHK-21, AT_{3}Neo and AT_{3}Bcl_{2} cell lines (Tables 4.1-4.3). TUNEL and immunocytochemical studies confirmed this property (Table 4.5). The results from the assays performed also illustrate the ability of the AT_{3}Bcl_{2} cells to delay the onset of apoptosis. Mock-infected BHK-21, AT_{3}Neo and AT_{3}Bcl_{2} control cells are negative for virus antigen and DNA fragmentation and therefore remained unstained as seen in Figure 4.17 parts A, B and C. A positive immunocytochemical and TUNEL assay was achieved when the BHK-21, AT_{3}Neo and AT_{3}Bcl_{2} cell lines were infected with the L10 and A7 virus strain, illustrated in Figure 4.17 parts D-G and H-K, respectively. BHK-21 and AT_{3}Neo cell lines infected with the M9 strain were positive for immunocytochemistry and TUNEL, displayed in Figure 4.17 parts L and M. However, AT_{3}Bcl_{2} cells infected with the M9 strain were negative for immunocytochemistry and TUNEL (Figure 4.17, part O). The maximum duration of the experiment post-infection was sixty hours. The AT_{3}Bcl_{2} cells infected with the M9 virus strain did not reach 25% CPE until after sixty hours (Table 4.3) and as such were undetectable by immunocytochemical and TUNEL analysis. TUNEL and immunocytochemistry analysis of AT_{3}Neo and AT_{3}Bcl_{2} (assayed at the p.i. time of 25% CPE for the AT_{3}Neo cells) cells demonstrates the delay of apoptosis conferred by the Bcl_{2}
Figure 4.17 TUNEL and immunocytochemistry analysis of SFV infected cells. All cells were infected with an MOI of 5 and fixed at 25% CPE, except for plates F, J and N which represent AT₃Bcl₂ cells fixed at the corresponding time of 25% CPE in AT₃Neo cells. A-C Mock-infected BHK-21, AT₃Neo and AT₃Bcl₂ cells respectively. D-G L10 infected BHK-21, AT₃Neo and AT₃Bcl₂ cells respectively. H-K A7 infected BHK-21, AT₃Neo and AT₃Bcl₂ cells respectively. L-O M9 infected BHK-21, AT₃Neo and AT₃Bcl₂ cells respectively. **NOTE:** The presence of brown staining in the cytoplasm, indicating positive immunocytochemistry, and the presence of blue/indigo staining in the nuclei, indicating a positive TUNEL reaction, in D, E, G, H, I, K, L and M.
4.2.2: AFM analysis of the emergence of progeny virus.

AFM studies were performed on BHK-21 cells infected with the TMEV strains and BHK-21, AT₃Neo and AT₃Bcl₂ cells infected with the SFV strains. Each virus infected cell sample and the respective controls were fixed at 25% CPE. All the samples were analysed with the AFM in contact mode. The experiments were performed in duplicate and three different areas in each sample were imaged to ensure that all possible features were observed. As for the AFM investigation of viral entry, roughness (Ra), topographic and line analysis of the samples was performed.

The mean Ra values for each virus and cell line are illustrated graphically in Figure 4.18. It would be expected that the Ra values for infected cells would increase with advancing levels of CPE due to the increasing levels of cell deterioration. An increased Ra is consistently seen at 25% CPE in the infected BHK-21 cells. L10 and A7 infected AT₃Neo cells have increased Ra values when compared to the mock-infected control, however M9 has a decreased Ra value. In the case of L10 infected AT₃Neo cells, the Ra value is higher than that of the L10 infected AT₃Bcl₂ cells fixed at the same time p.i. This indicates via Ra analysis that CPE is delayed in the AT₃Bcl₂ cells. M9 infected AT₃Neo cells have a slightly decreased Ra value compared to the control, and the M9 infected
Figure 4.18 Graph of roughness (Ra), from a user-defined area of infected and mock-infected cultured cells. BHK-21, AT₃Neo and AT₃Bcl₂ cell lines were infected with the appropriate viruses at an MOI of 5. The cells were fixed in 4% paraformaldehyde at 25% CPE (AT₃Bcl₂ cells were fixed at two time points, the first was the time p.i. at which the viruses induced 25% CPE in AT₃Neo cells and the second was the time p.i. at which the virus induced 25% CPE in AT₃Bcl₂ cells).
AT₃Bcl₂ cells fixed at 25% CPE of AT₃Neo cells is raised. Infected AT₃Bcl₂ cells at 25% CPE have increased Ra values in comparison to the mock-infected control. This again reveals that Ra values are higher in cells undergoing CPE.

Topographic and line analysis data for each virus infected cell line at 25% CPE is illustrated in Figures 4.19-4.23. In each Figure it is evident that the cells are undergoing cytopathic changes. Topographically, it can be seen that the infected cells are starting to lose their natural shape and round up and the cell numbers are sometimes decreased in comparison to the respective controls. However, virus particles cannot be determined from the data obtained. To address this, samples with increased levels of CPE were examined.

BHK-21 cells infected with the GDVII strain of TMEV were analysed at thirty-five, fifty and seventy-five percent CPE. Ra analysis of these samples is shown in Figure 4.24. The results of the Ra analysis were very inconsistent for these samples and few conclusions could be drawn. It is possible that at 50% CPE the increased Ra values are due to large amounts of virus being released by the cells. Figure 4.25 illustrates the topographic and line analysis data of the samples. From this data it is evident that the higher the level of CPE the more deteriorated the cells appear. However, no evidence of virus particles can be identified emerging from the cells.
Figure 4.19 Topographic and line analysis of mock-inf and TMEV infected BHK-21 cells. The cells were infected at a MOI of 5 and at 25% CPE. A= Mock-infected control, B-D= GDVII, DA and BeAn infected cells respectively.
Figure 4.20 Topographic and line analysis of mock-infected and SFV infected BHK-21 cells. The cells were infected at a MOI of 5 and fixed at 25% CPE. A= Mock-infected control, B-D= L10, M9 and A7 infected cells respectively.
Figure 4.21 Topographic and line analysis of mock-infected and SFV infected AT3Neo cells. The cells were infected at a MOI of 5 and fixed at 25% CPE. A= Mock-infected control, B= L10 infected cells and C= M9 infected cells.
Figure 4.22 Topographic and line analysis of mock-infected and SFV infected AT₃Bcl₂ cells. The cells were infected at a MOI of 5 and fixed at the time p.i. which the viruses had induced 25% CPE in AT₃Neo cells. A= Mock-infected control, B=L10 infected cells and C=M9 infected cells.
Figure 4.23 Topographic and line analysis of mock-infected and SFV infected AT₃Bcl₂ cells. The cells were infected at a MOI of 5 and fixed at the time p.i. of 25% CPE. A= Mock-infected control, B= L10 infected cells and C= M9 infected cells.
Figure 4.24 Graph of roughness (Ra), from a user-defined area of infected and mock-infected cultured cells. BHK-21 cells infected with GDVII virus (MOI of 5) and fixed at 35, 50 and 75% CPE.
Figure 4.25 Topographic and line analysis of mock-infected and GDVII infected BHK-21 cells. The cells were infected at a MOI of 5 and fixed at various levels of CPE. A= Mock-infected control, B-D= 35%, 50% and 75% CPE respectively.
To further investigate this aspect of the study, BHK-21 cells were infected with all of the virus strains and fixed at 75% CPE. The Ra analysis of these samples is illustrated in Figure 4.26. All the infected cells except for the virulent GDVII and L10 strains have raised Ra values in comparison to the mock-infected control. Topographic analysis revealed that the infected cells were exhibiting CPE (Figures 4.27 and 4.28). Line analysis of the samples (Figures 4.27 and 4.28) revealed that all the infected cells at 75% CPE have higher cell heights than the mock-infected controls. This may be due to aggregates of progeny virus, which are known to remain attached to the cell membrane on the extracellular side after they have exited through the membrane (Blasco and Moss, 1992, 1991; Payne and Kirstenson, 1982; Ohnesorge et al., 1997).

4.2.3: Summary of the analysis of the emergence of progeny virus.

The visualisation of progeny virus particles emerging from infected cells with the AFM was not achieved. However, the cytopathic effects of the viruses on the cells were observed especially when samples were fixed at 75% CPE. Increased Ra values were observed for most of the virus strains indicating that the surface of these cells was rougher than the mock-infected controls. This is probably due to cell deterioration of the infected cell surfaces due to CPE. Also, the cell heights of the infected cells were increased when compared to the mock-infected control and this effect may be due to aggregates of progeny virus.
Figure 4.26 Graph of roughness (Ra), from a user-defined area of infected and mock-infected cultured cells. BHK-21 cells infected with all six virus strains (MOI of 5) and fixed at 75% CPE. Note all the infected cells except for the virulent GDVII and L10 strains have raised Ra values in comparison to the control.
Figure 4.27 Topographic and line analysis of mock-infected and TMEV infected BHK-21 cells. The cells were infected at a MOI of 5 and fixed at 75% CPE. A= Mock-infected control, B-D= GDVII, DA and BeAn infected cells respectively.
Figure 4.28 Topographic and line analysis of mock-infected and SFV infected BHK-21 cells. The cells were infected at a MOI of 5 and fixed at 75% CPE. A= Mock-infected control, B-D= L10, M9 and A7 infected cells respectively.
Chapter 5

Viscoelasticity analysis of virally infected cultured cells
5.1: Introduction.

Viscoelasticity analysis of virally infected and mock-infected control cells is presented in this chapter. Viscoelasticity is a mechanical property of cells and it is indicative of the relative stiffness of biological material (Weyn et al., 1998; Lekka et al., 1999). This type of analysis was performed to investigate the possible occurrence of changes in relative local viscoelasticity of cells immediately post-infection (p.i.) and at the time of viral progeny release. Relative viscoelasticity values were calculated quantitatively using the force measurement software of the AFM. For a detailed description of the attainment of force measurements and their conversion into viscoelasticity values refer to Chapter 2, section 2.2.5. An increase in local cell viscoelasticity relative to mock-infected control cells indicates a softening of the virally-infected cells. Conversely, a decrease in relative local cell viscoelasticity indicates stiffening of virally infected cells.

Literature searches revealed three important factors that required consideration prior to the performance of force measurements on cultured cells:

The first is the location of the point on the cell surface from which the force measurement is obtained. The ideal location is one at which complete indentation of the cell can be achieved without interference from underlying stiff structures, the most common of which are the nucleus and the substrate on which the cells are cultured. As a result of this, force measurements obtained in this study were acquired from non-nuclear, non-border (to avoid low cell heights and subsequent
substrate interference) regions (see Figure 5.1) of the cultured cells (Weisenhorn et al., 1993; Ricci and Grattarola, 1994; Weyn et al., 1998).

The second factor that can have implications for viscoelasticity measurements is the hydration state of the cells. Dehydrated cells are much stiffer and as such have much lower viscoelasticity values than hydrated cells. Also, dehydrated cells show a uniform indentation profile over the whole cell that is independent of fixation. It was deemed necessary, therefore, for the cells to be in a hydrated state for the performance of force measurements (Weyn et al., 1998). Chapter 3 of this thesis reported on the optimisation of a cell culture fixation technique for AFM investigations. Post-fixation with the optimised technique, cultured cells remain in a hydrated state, therefore, this factor was not addressed further.

Finally, the fixation state of the cultured cells can have implications for viscoelasticity measurements. It has been reported that cultured cells undergo decreasing softness with increasing fixation strength (Hoh and Schoenenberger, 1994; Weyn et al., 1998). The optimised fixation technique determined in Chapter 3 of this study included the use of paraformaldehyde (4%). It was necessary to ensure that this concentration of paraformaldehyde did not cause too much stiffening of the cell samples, and subsequently obscure any possible differences in viscoelasticity measurements between infected and mock-infected cultured cells. Therefore, force measurements were performed on two and four percent paraformaldehyde fixed AT₃Neo cells. The viscoelasticity measurements
Figure 5.1 AFM image of BHK-21 cells. The three red rectangles superimposed on the image represent examples of non-nuclear, non-border positions from which force curve measurements were taken to determine relative local viscoelasticity.
obtained confirmed that the four percent paraformaldehyde-fixed cells were stiffer than the two percent paraformaldehyde-fixed cells (25 nN/nm and 65 nN/nm respectively). As the aim of this part of the study is to compare relative viscoelasticity measurements of infected and mock-infected cultured cells, the fixative strength is of little consequence once all the cell samples are treated with the same strength of fixative. Therefore, to maintain consistency throughout the study as a whole, the four percent paraformaldehyde fixative strength was used to fix the cultured cells prior to the performance of force measurements using the AFM.

The remainder of this chapter is divided into two sections. The first presents viscoelasticity measurements performed on cells imaged one hour p.i. (i.e. post-entry). The second section presents viscoelasticity measurements obtained during the subsequent release of progeny virus, the fixation times for which were chosen using data obtained in Chapter 4 (Tables 4.1-4.4).

5.2: Viscoelasticity analysis of viral entry.

BHK-21, AT₃Neo and AT₃Bcl₂ cells were infected with an avirulent and virulent strain of both TMEV (DA and GDVII) and SFV (A7 and L10) respectively. Cells were seeded onto 25 mm x 25 mm coverslips in 10 mm x 35 mm tissue culture dishes and subsequently infected with the appropriate virus strain at a multiplicity of infection (MOI) of ten. The infection was allowed to proceed for one hour, after which the cells were fixed in four percent paraformaldehyde and imaged with the
layered imaging mode of the AFM with a non-contact tip. Once a satisfactory image was obtained, three force measurements per sample were acquired from non-nuclear, non-border regions of the cells. The force measurements were converted to viscoelasticity values and a standard error of the mean viscoelasticity value was calculated for each sample and these values are illustrated in both tabular and graphical form in Figures 5.2-5.4.

5.2.1: Viscoelasticity analysis of viral entry in BHK-21 cells.
Viscoelasticity analysis results of virally infected (GDVII, DA, L10 and A7) and mock-infected control BHK-21 cells are illustrated in Figure 5.2. All the virally infected BHK-21 cells had higher mean viscoelasticity values than the mock-infected control cells, indicating that viral infection of this type of cultured cell leads to a softening of the cells. However, when the standard error of the mean was calculated it became evident that the range of the virally infected (with the exception of DA infected) sample error was overlapping that of the control. Thus no acceptable difference can be determined between viscoelasticity values of virally infected and mock-infected BHK-21 cells. DA infected BHK-21 cells had appreciably increased viscoelasticity values when compared to the control cells, indicating in this case that there was a softening of the infected cells in comparison to the mock-infected control cells.

BHK-21 cells infected with the avirulent strains (DA and A7) had higher mean viscoelasticity values than the virulent strains (GDVII and L10). This suggests
Figure 5.2 Viscoelasticity measurements of mock-infected and virally infected BHK-21 cells acquired during viral entry. BHK-21 cells were infected at a MOI of 10 with the virulent (TMEV strain GDVII, SFV strain L10) and avirulent (TMEV strain DA, SFV strain A7) strains of TMEV and SFV. Samples were fixed one hour post-infection with 4% paraformaldehyde and three force curve measurements per sample were performed and converted to relative viscoelasticity measurements. Subsequently, a mean viscoelasticity value and the standard error of the mean were calculated for each sample. A Illustrates in tabular form the mean viscoelasticity values obtained plus or minus the corresponding standard error of the mean. B Illustrates in graphical form the mean viscoelasticity values obtained plus or minus the corresponding standard error of the mean (error bars).
<table>
<thead>
<tr>
<th>BHK-21 cells</th>
<th>Viscoelasticity (nN/nm) (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock-infected control</td>
<td>266.5 ± 55</td>
</tr>
<tr>
<td>GDVII infected</td>
<td>365.2 ± 90</td>
</tr>
<tr>
<td>DA infected</td>
<td>1928 ± 740</td>
</tr>
<tr>
<td>L10 infected</td>
<td>386.8 ± 106</td>
</tr>
<tr>
<td>A7 infected</td>
<td>514.7 ± 224</td>
</tr>
</tbody>
</table>

**B**

![Graph showing viscoelasticity of BHK-21 cells infected with different strains]

Legend:
- Control (Mock-infected)
- GDVII infected
- DA infected
- L10 infected
- A7 infected
that infection with avirulent strains leads to a greater degree of softening of BHK-21 cells than infection with virulent strains. When the standard error of the mean was considered, however, it was evident that the range of error values for the A7 (avirulent) infected cells overlapped those of the virulent L10 strain. Alternatively, the range of error of the DA (avirulent) infected cells was outside the range of error of GDVII (virulent) infected cells. Therefore only in the case of TMEV infected BHK-21 cells can it be supposed that infection with an avirulent strain results in increased softening than infection with a virulent strain.

Mean viscoelasticity and standard error of the mean ranges revealed no differences between SFV (enveloped) and TMEV (non-enveloped) infected BHK-21 cells.

5.2.2: Viscoelasticity analysis of viral entry in AT₃Neo cells.

All the virally infected AT₃Neo cells had lower mean viscoelasticity values than the mock-infected control cells (Figure 5.3). Standard error of the mean analysis of the results confirmed this with the exception of DA infected cells. This suggests that viral infection of AT₃Neo cells with GDVII, L10 and A7 leads to stiffening of the AT₃Neo cells.

Mean viscoelasticity values and standard error of the mean ranges did not reveal any distinction between avirulently and virulently SFV infected AT₃Neo cells. However, in the case of TMEV infected AT₃Neo cells both the mean
Figure 5.3 Viscoelasticity measurements of mock-infected and virally infected AT₃Neo cells acquired during viral entry. AT₃Neo cells were infected at a MOI of 10 with the virulent (TMEV strain GDVII, SFV strain L10) and avirulent (TMEV strain DA, SFV strain A7) strains of TMEV and SFV. Samples were fixed one hour post-infection with 4% paraformaldehyde and three force curve measurements per sample were performed and converted to relative viscoelasticity measurements. Subsequently, a mean viscoelasticity value and the standard error of the mean were calculated for each sample. A Illustrates in tabular form the mean viscoelasticity values obtained plus or minus the corresponding standard error of the mean. B Illustrates in graphical form the mean viscoelasticity values obtained plus or minus the corresponding standard error of the mean (error bars).
<table>
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<td>236 ± 50</td>
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<tr>
<td>DA infected</td>
<td>436.7 ± 25</td>
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<tr>
<td>L10 infected</td>
<td>229.7 ± 59</td>
</tr>
<tr>
<td>A7 infected</td>
<td>198.7 ± 6</td>
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</table>

A

B

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viscoelasticity values and standard error of the mean ranges revealed that the virulent (GDVII) strain did produce a greater degree of stiffening than the avirulent (DA) strain.

Mean viscoelasticity and standard error of the mean ranges revealed no differences between SFV (enveloped) and TMEV (non-enveloped) infected AT₃Neo cells.

5.2.3: Viscoelasticity analysis of viral entry in AT₃Bcl₂ cells.

GDVII, DA and L10 infected AT₃Bcl₂ cells had lower mean viscoelasticity values than the mock-infected control indicating that infection of AT₃Bcl₂ cells with these virus strains leads to a stiffening of the cells (Figure 5.4). A7 infection of AT₃Bcl₂ cells had the opposite effect on the infected cells mean viscoelasticity values with the infected cells appearing softer than the mock-infected control. However, the standard error of the mean ranges for all the virally infected cells overlapped with the range of the mock-infected control AT₃Bcl₂ cells and thus no significant difference between virally infected and mock-infected control cells was observed.

Viscoelasticity analysis of SFV and TMEV infected AT₃Bcl₂ cells did not reveal any distinction between avirulently and virulently infected cells.
Figure 5.4 Viscoelasticity measurements of mock-infected and virally infected AT$_3$Bcl$_2$ cells acquired during viral entry. AT$_3$Bcl$_2$ cells were infected at a MOI of 10 with the virulent (TMEV strain GDVII, SFV strain L10) and avirulent (TMEV strain DA, SFV strain A7) strains of TMEV and SFV. Samples were fixed one hour post-infection with 4% paraformaldehyde and three force curve measurements per sample were performed and converted to relative viscoelasticity measurements. Subsequently, a mean viscoelasticity value and the standard error of the mean were calculated for each sample. A Illustrates in tabular form the mean viscoelasticity values obtained plus or minus the corresponding standard error of the mean. B Illustrates in graphical form the mean viscoelasticity values obtained plus or minus the corresponding standard error of the mean (error bars).
<table>
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<th>AT₃Bcl₂ cells</th>
<th>Viscoelasticity (nN/nm) (Mean ± SEM)</th>
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<tbody>
<tr>
<td>Mock-infected control</td>
<td>527.3 ± 172</td>
</tr>
<tr>
<td>GDVII infected</td>
<td>500 ± 177</td>
</tr>
<tr>
<td>DA infected</td>
<td>391.7 ± 111</td>
</tr>
<tr>
<td>L10 infected</td>
<td>470.2 ± 235</td>
</tr>
<tr>
<td>A7 infected</td>
<td>1599 ± 1000</td>
</tr>
</tbody>
</table>

A

B
Mean viscoelasticity and standard error of the mean ranges revealed no differences between SFV (enveloped) and TMEV (non-enveloped) infected AT\textsubscript{3}Bcl\textsubscript{2} cells.

5.2.4: Discussion of viscoelasticity analysis of viral entry.

When the viscoelasticity results for viral infection of all three cell types (BHK-21, AT\textsubscript{3}Neo and AT\textsubscript{3}Bcl\textsubscript{2}) were examined, no consistent effect on cell viscoelasticity was apparent immediately post viral entry. No difference in cell viscoelasticity was noted when BHK-21 cells were infected with the virus strains, with the exception of DA infected cells which were softer than the mock-infected control cells. Similarly, no difference was observed when AT\textsubscript{3}Bcl\textsubscript{2} cells were virally infected. Virally infected AT\textsubscript{3}Neo cells, with the exception of DA infected cells, had decreased viscoelasticity values compared to the mock-infected control indicating that these virus strains induced cell stiffening during viral entry. This result would indicate that the GDVII strain of TMEV was entering the AT\textsubscript{3}Neo cells and that this strain does not induce cytopathic effects (CPE) (Chapter 4, Table 4.2) in AT\textsubscript{3}Neo cells due to a block in replication post-infection.

Viscoelasticity analysis of avirulent (A7) and virulent (L10) SFV strain infection of BHK-21, AT\textsubscript{3}Neo and AT\textsubscript{3}Bcl\textsubscript{2} cells revealed no differentiation in viscoelasticity terms between avirulently and virulently infected cells. Likewise, no difference in viscoelasticity values was noted for avirulently (DA) and virulently (GDVII) TMEV infected AT\textsubscript{3}Bcl\textsubscript{2} cells. However, avirulently (DA) and virulently (GDVII) TMEV
infected BHK-21 and AT₃Neo cells revealed that cells infected with the avirulent strain were relatively softer than the virulently infected cells.

Viscoelasticity analysis of viral entry into all three cell lines (BHK-21, AT₃Neo and AT₃Bcl₂) did not reveal any difference between enveloped (SFV) and non-enveloped (TMEV) infected cells. This would indicate that non-enveloped and enveloped viruses do not have conflicting effects on relative cell viscoelasticity at the stage of viral entry.

5.3: Viscoelasticity analysis of viral emergence.

The time point post-infection of 50% CPE (Chapter 4, Tables 4.1-4.3) was chosen to attempt to detect changes in viscoelasticity during the emergence of progeny virus. BHK-21 cells were infected with both TMEV (GDVII and DA) and SFV (L10 and A7). Alternatively, AT₃Neo and AT₃Bcl₂ cells were infected solely with SFV (L10 and A7). TMEV infection of AT₃Neo and AT₃Bcl₂ cells was not performed as it was determined previously in Chapter 4 of this study that TMEV infection of these cell types does not result in CPE i.e TMEV infection of these cell types is not successful and no progeny virus is released. Cells were infected with the appropriate virus strain with a MOI of five, the infection was allowed to proceed until 50% CPE occurred and the cells were fixed in 4% paraformaldehyde. Post-fixation, the cells were imaged with the layered imaging mode of the AFM with a non-contact tip. Once a satisfactory image was obtained three force measurements per sample were acquired from non-nuclear, non-
border regions of the cells. The force measurements were converted to viscoelasticity values and a standard error of the mean viscoelasticity value was calculated for each sample and these values are illustrated in both tabular and graphical form in Figures 5.5-5.7.

5.3.1: Viscoelasticity analysis of viral emergence from BHK-21 cells.
BHK-21 cells infected with the GDVII, DA and L10 viruses at 50% CPE had mean viscoelasticity values greater than the mock-infected control cells (Figure 5.5). This indicated that at 50% CPE the infected cells were softer than the control cells. Mean viscoelasticity values of A7 infected BHK-21 cells were lower than those of the mock-infected control, indicating that at 50% CPE A7 infected cells were stiffer than the control cells. The standard error of the mean range for the DA infected BHK-21 cells and the mock-infected control cells did not overlap. However, the standard error of the mean ranges for the mock-infected control cells and the GDVII, L10 and A7 infected BHK-21 were overlapping.

50% CPE induced by the virulent strain of TMEV (GDVII) resulted in decreased mean viscoelasticity values in comparison to the avirulently (DA) infected cells. This revealed that the virulently infected cells were softer than the avirulently infected cells. The standard error of the mean ranges, however, were overlapping. The opposite effect was observed when BHK-21 cells at 50% CPE induced by SFV were examined. The mean viscoelasticity values for the
Figure 5.5 Viscoelasticity measurements of mock-infected and virally infected BHK-21 cells acquired during the emergence of viral progeny. BHK-21 cells were infected at a MOI of 10 with the virulent (TMEV strain GDVII, SFV strain L10) and avirulent (TMEV strain DA, SFV strain A7) strains of TMEV and SFV. Samples were fixed at 50% CPE with 4% paraformaldehyde and three force curve measurements per sample were performed and converted to relative viscoelasticity measurements. Subsequently, a mean viscoelasticity value and the standard error of the mean were calculated for each sample. A Illustrates in tabular form the mean viscoelasticity values obtained plus or minus the corresponding standard error of the mean. B Illustrates in graphical form the mean viscoelasticity values obtained plus or minus the corresponding standard error of the mean (error bars).
<table>
<thead>
<tr>
<th>BHK-21 cells</th>
<th>Viscoelasticity (nN/nm) (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock-infected control</td>
<td>345 ± 42</td>
</tr>
<tr>
<td>GDVII infected</td>
<td>574.5 ± 287</td>
</tr>
<tr>
<td>DA infected</td>
<td>955 ± 207</td>
</tr>
<tr>
<td>L10 infected</td>
<td>437.5 ± 219</td>
</tr>
<tr>
<td>A7 infected</td>
<td>138 ± 138</td>
</tr>
</tbody>
</table>

**Figure A**

**Figure B**
virulently (L10) infected cells were higher than those of the avirulently (A7) infected cells, however the standard error of the mean ranges were overlapping.

At 50% CPE the enveloped SFV's caused decreased mean cell viscoelasticity values compared to the TMEV infected cells. The standard error of the mean ranges of the SFV and TMEV infected samples were however overlapping, except for the A7 infected BHK-21 cells in comparison to both the TMEV infected samples. This result suggested that enveloped A7 progeny viruses cause increased stiffening of the cells in comparison to the non-enveloped TMEV's.

5.3.2: Viscoelasticity analysis of viral emergence from AT₃Neo cells.

The mean viscoelasticity values obtained from AT₃Neo cells at 50% CPE induced by the SFV L10 and A7 strains were lower than that of the mock-infected control cells (Figure 5.6). This indicated that the virus emergence from AT₃Neo cells had a stiffening effect on the cells. The standard error of the mean range for the A7 infected cells did fall within the mock-infected control range, however the range of the L10 values was outside that of the control. Therefore the emergence of L10 progeny virus from the AT₃Neo cells did result in stiffening of the cells.

The virulently (L10) infected AT₃Neo cells mean viscoelasticity values were lower than that of the avirulently (A7) infected cells, this indicated that the virulently infected cells were stiffer than the avirulently infected cells. This observation was tenuous however, as the standard error of the mean values were overlapping.
Figure 5.6 Viscoelasticity measurements of mock-infected and virally infected AT₃Neo cells acquired during the emergence of viral progeny. AT₃Neo cells were infected at a MOI of 10 with the virulent (L10) and avirulent (A7) strains of SFV. Samples were fixed at 50% CPE with 4% paraformaldehyde and three force curve measurements per sample were performed and converted to relative viscoelasticity measurements. Subsequently, a mean viscoelasticity value and the standard error of the mean were calculated for each sample. A Illustrates in tabular form the mean viscoelasticity values obtained plus or minus the corresponding standard error of the mean. B Illustrates in graphical form the mean viscoelasticity values obtained plus or minus the corresponding standard error of the mean (error bars).
<table>
<thead>
<tr>
<th>AT₃Neo cells</th>
<th>Viscoelasticity (nN/nm) (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock-infected control</td>
<td>464 ± 153</td>
</tr>
<tr>
<td>L10 infected</td>
<td>234.5 ± 67</td>
</tr>
<tr>
<td>A7 infected</td>
<td>298 ± 122</td>
</tr>
</tbody>
</table>

![Graph showing viscoelasticity of AT₃Neo cells](image-url)
5.3.3: Viscoelasticity analysis of viral emergence from AT\textsubscript{3}Bcl\textsubscript{2} cells.

Viscoelasticity analysis of SFV induced 50% CPE in AT\textsubscript{3}Bcl\textsubscript{2} cells revealed that the infected (L10 and A7) cells had lower mean viscoelasticity values compared to the mock-infected control (Figure 5.7). The standard error of the mean ranges of the infected cells were outside the mock-infected control range. This indicated that the emergence of L10 and A7 progeny virus from AT\textsubscript{3}Bcl\textsubscript{2} cells resulted in cell stiffening.

The avirulently (A7) infected AT\textsubscript{3}Bcl\textsubscript{2} cells had a higher mean viscoelasticity value than the virulently (L10) infected cells, indicating that the avirulently infected cells were softer than the virulently infected cells at 50% CPE. However, the standard error of the mean ranges of the L10 and A7 viscoelasticity values did overlap.

5.3.4: Discussion of viscoelasticity analysis at viral emergence.

Viscoelasticity analysis of the emergence of viral progeny from BHK-21 cells demonstrated that the egress of the non-enveloped DA virions resulted in the softening of the cells. No significant difference in viscoelasticity measurements was observed during the analysis of L10, A7 and GDVII progeny virion emergence from infected BHK-21 cells. Viscoelasticity analysis revealed no notable differences between the egress of avirulent and virulent virions from BHK-21 cells. Comparison of the viscoelasticity values of non-enveloped (TMEV) and enveloped (SFV) viral emergence from BHK-21 cells revealed that the A7
Figure 5.7 Viscoelasticity measurements of mock-infected and virally infected AT$_3$Bcl$_2$ cells acquired during the emergence of viral progeny. AT$_3$Bcl$_2$ cells were infected at a MOI of 10 with the virulent (L10) and avirulent (A7) strains of SFV. Samples were fixed at 50% CPE with 4% paraformaldehyde and three force curve measurements per sample were performed and converted to relative viscoelasticity measurements. Subsequently, a mean viscoelasticity value and the standard error of the mean were calculated for each sample. A Illustrates in tabular form the mean viscoelasticity values obtained plus or minus the corresponding standard error of the mean. B Illustrates in graphical form the mean viscoelasticity values obtained plus or minus the corresponding standard error of the mean (error bars).
### AT3Bcl2 cells

<table>
<thead>
<tr>
<th></th>
<th>Viscoelasticity (nN/nm) (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock-infected control</td>
<td>1714 ± 953</td>
</tr>
<tr>
<td>L10 infected</td>
<td>408 ± 106</td>
</tr>
<tr>
<td>A7 infected</td>
<td>469 ± 158</td>
</tr>
</tbody>
</table>

#### A

![Graph showing viscoelasticity](image)

- **Control (Mock-infected)**
- **L10 infected**
- **A7 infected**

#### B

185
strain of SFV caused increased stiffening of infected cells relative to both of the TMEV strains.

Viscoelasticity analysis of SFV emergence from AT₃Neo cells revealed that the egress of L10 progeny virus has a stiffening effect on the infected cells. No appreciable difference was observed between the emergence of avirulent (A7) and virulent (L10) progeny virions from AT₃Neo cells with respect to viscoelasticity measurements.

The egress of L10 and A7 progeny viruses from AT₃Bcl₂ cells resulted in decreased viscoelasticity values compared to the mock-infected control, thus revealing that the release of L10 and A7 virions from AT₃Bcl₂ cells results in cell stiffening. No significant viscoelastic difference was observed between the emergence of virulent (L10) and avirulent (A7) viruses from AT₃Bcl₂ cells.

The emergence of enveloped progeny SFV's in comparison to the mock-infected controls revealed that the virally infected cells were stiffer in a number of cases (L10 infected AT₃Neo cells; L10 and A7 infected AT₃Bcl₂ cells). This effect may reflect the emergence strategy of enveloped viruses in that the cell membrane is being removed by the egressing virus thereby causing an increase in cell volume: cell surface ratio which may be resulting in the cells becoming more compact and firm.
Chapter 6

AFM analysis of virus particles
The use of atomic force microscopy to study viruses offers the prospect of studying in detail, at high resolution, the shape of the virus particle and the characteristics of its surface. A second application of this instrument is the investigation of the effects of antibodies, anti-viral drugs, chemicals and pharmaceuticals etc. on viruses. In this chapter the conditions of virus imaging were optimised. Following this, a non-enveloped TMEV (strain DA) and an enveloped SFV (strain L10) were imaged using the AFM.

6.1: Optimisation of virus imaging.

Silicon wafers, which have an extremely flat surface, were used as the matrix on which virus particles were immobilised. The wafers were washed and silanised prior to virus isolation. A sulphochromic wash was employed to remove organic contamination without damaging the oxide layer of the wafer surface. The sulphochromic wash also produces a hydrophilic surface as it creates hydroxyl groups on the silica by opening siloxane bonds. Silanisation of the wafer surface converts this hydrophilic surface to a highly hydrophobic surface.

Initial virus isolation was performed by applying undiluted, purified virus particles on a washed and silanised wafer. All preliminary investigations were performed with the SFV L10 strain. The virus particles were allowed to sediment and adhere to the wafer surface for 2 hours. The wafer was then washed in 0.45 μm filtered distilled water, dried under nitrogen gas and imaged using the AFM in contact mode. Images obtained revealed the presence of structures on the wafer surface.
(Figure 6.1). Line analysis of the structures revealed that they were uniform in width (582 nm, n=7) and height (34 nm, n=7), however, it was impossible to definitively identify these structures as virus particles.

In an attempt to identify the structures seen on the above image, the number of virus particles applied to the wafer surface was increased. To achieve this, the amount of purified virus applied to the wafer surface to attain a monolayer was calculated (Appendix C), and this amount was applied to the surface of a washed and silanised wafer. The virus solution was allowed to evaporate overnight and the sample was then imaged using the AFM in contact mode. Putative virus structures were again observed on the wafer surface and a force curve measured on one of the observed structures revealed adhesion between the tip and the proposed virus particle. This adhesion can lead to distortion of “true” image data, therefore the contact mode was deemed unsuitable for further imaging and the sample was re-imaged using the AFM in non-contact mode. Non-contact images of the L10 virus monolayer revealed what appeared to be single and aggregated virus particles (Figure 6.2). Although the putative single virus particles were uniform in size (730 nm in width and 185 nm in height, n=6), it was impossible to definitively identify the structures as virus particles.

To definitively identify the structures as virus particles an antibody-antigen capture system was devised (Figure 6.3). In this system, an anti-virus antibody monolayer was used to capture virus (antigen) particles. Silicon wafers were
Figure 6.1 2D topographic image of SFV strain L10. The image revealed the presence of structures (arrowed) on the wafer surface. Line analysis of the structures revealed that they were uniform in width (582 nm, n=7) and height (34 nm, n=7), however, it was impossible to definitively identify these structures as virus particles.
Figure 6.2 A silicon wafer with a monolayer of L10 virus particles was imaged using the AFM in non-contact mode. A 2D topographic representation of the image obtained. Single (arrow) and aggregated (broken arrow) virus particles appear to be present on the wafer surface. B 3D presentation of the 2D image shown in part A.
Figure 6.3 Illustration of the antibody-antigen capture system employed to definitively identify virus particles. In this system a monolayer of anti-virus antibody molecules was applied to the sulfochromic washed and silanised wafer surface. The antibody monolayer was then used to capture its corresponding antigen i.e. virus particles.
washed and silanised as previously described and an antibody monolayer applied. The concentration of protein in the antibody stock solutions was determined using the Warburg and Christian (1942) method of protein determination and the stock solutions were diluted to 20 μg/ml in 0.1M citrate acetate buffer pH 5.

An antibody concentration (20 μg/ml), which ensured a monolayer was employed, as a sub-monolayer would result in sub-maximal virus particle capture and more importantly, tip contamination when the tip imaged between the individual antibody particles (Figure 6.4). The antibody concentration employed may have resulted in the presence of excess antibody structures which bound to monolayer antibodies rather than the antibody saturated silicon surface. These excess antibodies, which are weakly bound in comparison to silicon-bound antibodies, can detach during imaging and lead to tip contamination. In order to remove excess antibodies, the wafers were washed vigorously following antibody incubation, in phosphate buffered saline containing 0.1% Tween (PBST).

At a pH close to but not equal to the isoelectric point (pI) of IgG antibodies (pI of 7-8), the hydrophobic groups of the antibody structure are exposed (Bagchi and Birhaum, 1981). This serves to promote protein adsorption via hydrophobic reactions that occur between the antibody and the silanised wafer surface. In addition, at a slightly acidic pH (pH 5), proteins are prone to a greater level of hydrophobic exposure in the Fc portion, which contains antibody effector
Figure 6.4 Illustration of the effects of different antibody concentrations. A The concentration of antibody applied ensured an antibody monolayer on the wafer surface. B The antibody concentration applied resulted in a sub-monolayer of antibody on the wafer surface. This leads to sub-maximal virus capture and as the tip images in between the antibody particles it becomes contaminated. C The concentration of antibody applied leads to an excess of antibody particles which bind to monolayer antibodies when the silicon surface is antibody saturated. Excess antibodies are weakly bound and can detach during imaging resulting in tip contamination. Excess antibodies can be removed by vigorous washing in PBST.
functions of the antibody structure, than the Fab portions, which contain the antigen binding sites. This results in a site-directed orientation of the antibodies on the wafer surface (Figure 6.5), where the antibodies are preferentially adsorbed via the Fc portions onto the wafer surface. However, previous studies with silane surfaces have revealed that the adsorption of antibody molecules onto a silane surface results in a "side-on" orientation rather than a Fc site-directed orientation (Figure 6.5, part D) (Perrin et al., 1999).

Preliminary antibody monolayer experiments were performed with anti-TMEV antibody. This antibody was diluted as described above, applied to the wafer surface and incubated for 2 hours at 37°C in a humidified chamber. Subsequently the wafer was washed in PBST, then washed in 0.45 μm filtered distilled water, dried under nitrogen gas and imaged using the AFM in non-contact mode. The image obtained revealed an antibody monolayer (Figure 6.6). The height of the antibody monolayer was determined to be 5.21 nm (n=5) and its roughness (Ra) to be 2.98 nm. Previous studies revealed that anti-αfetoprotein antibody monolayers had a mean height of 5-8 nm and a roughness value of 1.19 nm (O'Connell, 1998). The height and roughness values of the anti-TMEV antibody monolayer were therefore comparable to those obtained previously for anti-αfetoprotein.

Having successfully obtained an antibody monolayer, the issue of virus immobilisation was addressed. Undiluted, purified virus was applied to an
Figure 6.5 Antibody orientations on silicon wafer surfaces. A The basic structure of an antibody molecule. Each antibody has one Fc (effector function) portion and two Fab (antigen binding) portions. B Both the Fc and Fab portions of the antibody molecule are partially denatured, exposing hydrophobic areas, to the same degree by a pH close to the antibody pi. Therefore they have equal attraction for the wafer surface, resulting in some antibodies binding by the Fab portions, this reduces the number of antigen binding sites available to react with virus particles. C At a slightly acidic pH a greater level of denaturation occurs in the Fc portion of the antibody molecule than in the Fab portion and this results in a site-directed orientation of the antibodies on the wafer surface. D The adsorption of antibody molecules onto a silane surface results in a "side-on" orientation rather than a Fc site-directed orientation.
Figure 6.6 Anti-TMEV antibody coated silicon wafer imaged using the AFM in non-contact mode. A 2D topographic image of the antibody layer, some holes in the monolayer were evident (arrows). B 3D presentation of the same image. The antibody layer was determined to be 5.21 nm (n=5) in height and had an Ra value of 2.98 nm.
antibody-covered wafer, which was incubated for 1.5 hours at room temperature, washed in PBST (to remove any loosely bound matter), then washed in 0.45 μm filtered distilled water, dried under nitrogen gas and imaged using the AFM in non-contact mode.

Four antibody:antigen samples were prepared, these included anti-TMEV:TMEV strain DA, anti-A5: SFV strain L10 virus capsids, anti-E1:SFV strain L10 and anti-E2:SFV strain L10 (Table 6.1). Neutralisation assays were performed to ensure that the antibody:antigen reactions were occurring (Table 6.2). Results of the anti-TMEV and TMEV strain DA neutralisation assay revealed that anti-TMEV neutralised the purified DA virus at antibody dilution $10^{-3}$ (n=2). SFV anti-A5 did not neutralise the purified L10 strain (n=3), this result was as expected as anti-A5 is a capsid directed antibody and as such will have no neutralising effect on enveloped virus particles. Anti-E1 had a neutralising effect on the purified SFV strain L10, neutralisation occurred at antibody dilution $10^{-3}$ (n=2). Likewise the anti-E2 had a neutralisation effect on purified SFV strain L10, neutralisation occurred at antibody dilution $10^{-3}$ (n=2).

Prior to virus particle analysis the AFM was calibrated with a TGT01 calibration grating (Ultrasharp, Moscow, Russia) to ensure that the instrument was reading accurately in the z (height) range.
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Antibody description</th>
<th>Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMEV</td>
<td>Polyclonal rabbit</td>
<td>Capsid protein</td>
</tr>
<tr>
<td>Anti-TMEV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SFV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-A5</td>
<td>Polyclonal rabbit</td>
<td>A5 capsid protein</td>
</tr>
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<td>Monoclonal mouse</td>
<td>E1 envelope protein</td>
</tr>
<tr>
<td>Anti-E2</td>
<td>Monoclonal mouse</td>
<td>E2 envelope protein</td>
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</tbody>
</table>

**Table 6.1** Summary of the antibodies used to isolate virus particles for AFM analysis and in the neutralisation assays. For example anti-A5 was used to isolate SFV strain L10 nucleocapsids and was a polyclonal rabbit antibody directed against the SFV A5 capsid protein.
<table>
<thead>
<tr>
<th>Virus</th>
<th>Antibody</th>
<th>Neutralisation</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMEV strain DA</td>
<td>Anti-TMEV</td>
<td>Yes</td>
</tr>
<tr>
<td>SFV strain L10</td>
<td>Anti-A5</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>Anti-E1</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Anti-E2</td>
<td>Yes</td>
</tr>
</tbody>
</table>

**Table 6.2** Neutralisation assay results (n=2). Each antibody had neutralisation effect except for anti-A5, which was an anti-capsid antibody, and therefore incapable of neutralising enveloped L10 virus particles.
6.2: Virus particle analysis.

AFM analysis of the TMEV strain DA immobilised with anti-TMEV resulted in successful imaging of virus structures (Figure 6.7). Two types of structures were evident and were postulated to be single and aggregated virus particles. Line analysis of the single virus structures revealed that the mean structure width and height were 446 nm and 15 nm (n=17), respectively. Two aggregated virus structures were observed with width and height readings of 432 nm and 59 nm (n=2) (Figure 6.7, labelled 1) and 546 nm and 27 nm (n=2) (Figure 6.7, labelled 2), respectively.

SFV strain L10 virus particles were isolated with a number of different antibodies. L10 virus capsids, revealed by removing the viral envelope, were immobilised with SFV anti-A5. Enveloped L10 virus particles were immobilised with SFV anti-E1 and anti-E2 which are directed against the E1 and E2 viral envelope proteins, respectively.

L10 capsid imaging (Figure 6.8) was performed and the images obtained revealed mostly single virus structures of 218 nm in width and 13 nm in height (n=30). One possible aggregation of virus particles (Figure 6.8, broken arrow) was observed, the dimensions of which were 458 nm in width and 191 nm in height (n=2).
Figure 6.7 AFM analysis of the TMEV strain DA immobilised with anti-TMEV. A 2D topographic representation of the image obtained. Two types of structures were evident and were postulated to be single (arrow) and aggregated (broken arrow) virus particles. Line analysis of the single virus structures revealed that the mean structure width and height were 446 nm and 15 nm (n=17), respectively. Two aggregated virus structures were observed with width and height readings of 432 nm and 59 nm (n=2) (labelled 1) and 546 nm and 27 nm (n=2) (labelled 2), respectively. B 3D presentation of the 2D image shown in part A.
A

B

203
Figure 6.8 AFM images of L10 virus capsids revealed by removing the virus envelope and immobilised with anti-A5. A 2D topographic representation of the image obtained. The image revealed single (arrow) virus structures and one possible aggregate (broken arrow) of virus particles. The single virus structure dimensions were 218 nm in width and 13 nm in height (n=30). One possible aggregation of virus particles was observed the dimensions of which were 458 nm in width and 191 nm in height (n=2). B 3D presentation of the 2D image shown in part A.
AFM imaging of enveloped L10 virus particles immobilised with anti-E1 revealed both single and aggregated virus particles (Figure 6.9). Line analysis measurements of single enveloped virus particles indicated that the particles were 160 nm in width and 28 nm in height (n=21). Three areas of aggregated virus were observed, the dimensions of which were 470 nm and 81 nm (n=2) (Figure 6.9, labelled 1), 285 nm and 60 nm (n=2) (Figure 6.9, labelled 2) and 257 nm and 48 nm (Figure 6.9, labelled 3) in width and height respectively.

Enveloped L10 virus particles were also immobilised with anti-E2 and images obtained, again, revealed both single and aggregated virus particles (Figure 6.10). The dimensions of the single virus particles imaged were determined by line analysis to be 164 nm in width and 36 nm in height (n=24). Three areas of virus aggregation were observed, these aggregates had dimensions of 402 nm and 75 nm (n=2) (Figure 6.10, labelled 1), 288 nm and 69 nm (n=2) (Figure 6.10, labelled 2) and 398 nm and 82 nm (n=2) (Figure 6.10, labelled 3) in width and height, respectively.

6.3: Interpretation of virus particle analysis.

Electron micrographs and x-ray diffraction analysis have revealed that picornaviruses are 24-30 nm in diameter (Rueckert, 1996). Analysis of AFM imaged DA virus particles revealed that the immobilised particles were 446 nm in width and 15 nm in height. These dimensions are approximately 15 times wider and \( \frac{1}{2} \) under height. Line analysis measurements of the two aggregates
Figure 6.9 AFM imaging of enveloped L10 virus particles immobilised with anti-E1. A 2D topographic representation of the image obtained. The image revealed both single (arrow) and aggregated (broken arrow) virus particles. Line analysis measurements of single enveloped virus particles indicated that the particles were 160 nm in width and 28 nm in height (n=21). Three areas of aggregated virus were observed, the dimensions of which were 470 nm and 81 nm (n=2) (labelled 1), 285 nm and 60 nm (n=2) (labelled 2) and 257 nm and 48 nm (n=2) (labelled 3) in width and height respectively. B 3D presentation of the 2D image shown in part A.
Figure 6.10 AFM images of enveloped L10 virus particles immobilised with anti-E2. A 2D topographic representation of image obtained. The image obtained revealed both single (arrow) and aggregated (broken arrow) virus particles. The dimensions of the single virus particles imaged were determined by line analysis to be 164 nm in width and 36 nm in height (n=24). Three areas of virus aggregation were observed, these aggregates had dimensions of 402 nm and 75 nm (n=2) (labelled 1), 288 nm and 69 nm (n=2) (labelled 2) and 398 nm and 82 nm (n=2) (labelled 3) in width and height, respectively. B 3D presentation of the 2D image shown in part A.
observed were interesting in that the heights of both were almost 4 times (59 nm) and twice (27 nm) the height of the single virus particles observed (15 nm). The aggregates were slightly less wide (432 nm), or \(\frac{1}{4}\) wider (546 nm), respectively, than the single virus particles (446 nm). The aggregate with the dimensions 432 nm x 59 nm may be composed of four single virus particles stacked on top of each other (Figure 6.11, part A), this particle conformation seems atypical and a more likely conformation was observed with the second aggregate. In this case the aggregate dimensions were 546 nm and 27 nm, these dimensions may be representative of two virus particles both attached to the antibody monolayer with one positioned slightly under the other (Figure 6.11, part B).

L10 virus capsids theoretically are spherical and 46 nm in diameter (Vogel et al., 1986). Single L10 virus capsid particle measurements were determined to be 218 nm in width and 13 nm in height, which is approximately 5 times as wide and \(\frac{1}{3}\) the height of the theoretical values. The aggregate of capsid structures observed were approximately twice as wide (458 nm) and 14 times higher (191 nm) than the single capsid dimensions determined using the AFM. It is possible that the capsids are forming a square base (2 capsids in width and 2 in breadth) which has numerous layers of capsid squares on top (Figure 6.11, part C).

Enveloped L10 virus particles theoretically are spherical and 64 nm in diameter (Vogel et al., 1986). Single enveloped virus particles immobilised with anti-E1 were approximately twice the width (160 nm) and \(\frac{1}{2}\) the height (28 nm) of the
Figure 6.11 Illustration of virus particle conformations. A Four virus particles in vertical formation. B Two virus particles both attached to the antibody monolayer, with one positioned slightly under the other. C Virus particles in a square base formation (top view) which can have numerous layers of virus squares layered top (lateral view). D Three tier pyramid virus particle formation. E Two tier pyramid virus particle formation.
theoretical dimensions. Aggregates observed were either 3 times (470 nm wide and 81 nm high) or twice the width and height (285 nm and 257 nm; 60 nm and 48 nm, respectively) of the single enveloped virus particle dimensions as determined by AFM analysis. In the aggregate which was 3 times the width and height the enveloped virus particles are likely to be arranged in a pyramid formation with 3 particles at the base, 2 in the second tier and 1 at the top (Figure 6.11, part D). The 2 aggregates with dimensions twice the width and height are also likely to be in a pyramid formation. In this case, however, the pyramid is composed of 2 tiers with 2 virus particles at the base and 1 virus particle in the top tier (Figure 6.11, part E).

Enveloped L10 virus particles were also immobilised using anti-E2. Single virus particles isolated in this manner had dimensions of 164 nm in width and 36 nm in height. These measurements are approximately 2½ times the width and ½ the height of the theoretical dimensions. All 3 aggregates of virus particles were approximately twice the width (402 nm, 288 nm and 398 nm) and twice the height (75 nm, 69 nm and 82 nm, respectively) of the single virus particle dimensions determined by AFM analysis. These values indicated that the virus particles were in a pyramid formation with 2 virus particles at the base and 1 virus particle lying on top (Figure 6.11, part E).

AFM analysis of DA capsids, L10 capsids and enveloped L10 viruses has revealed that measurements of the particles as determined by the AFM are wider
and lower in comparison to the theoretical virus dimensions. The spherical structure of the virus particle and the geometry of the AFM tip can lead to tip-particle convolution, the end effect of which is broadening of the structure being imaged (Putman et al., 1993). Tip broadening does not affect the measured height of a particle providing that the tip has access to one side of the structure. If the AFM was under-measuring in the z (height) range, the width of the particles imaged with a long-range scanner should not be affected. However, if a short-range tube scanner, in which the x, y and z direction movements are controlled by a common piezo, is used, under-measuring in the z direction will coincide with under-measuring in the x and y directions. Before AFM imaging of the samples was performed the instrument was calibrated and the tip geometry imaged using scanning electron microscopy. The AFM was found to be measuring accurately in the z direction and the tip geometry was revealed to be acceptable. Therefore the dimensions of the virus particles as determined by the AFM can be considered to be accurate.

The increase in width and decrease in height of virus particles may be a result of the particles collapsing during immobilisation. It was postulated that this increase in particle width and decrease in particle height was the result of virus particles collapsing from a sphere to an ellipsoid without a change in volume (Figure 6.12). If virus particle volume (V) is conserved then:

\[ V = \frac{4}{3}(\pi r^3) = \frac{4}{3}(\pi abc) = \frac{4}{3}(\pi a^2c) \]
Figure 6.12 Illustration of virus particles collapsing from a sphere to an ellipsoid. The volume of the sphere and the ellipsoid are the same, however the width and height of the ellipsoid are greater and less, respectively, than the sphere. $2r$ represents the diameter of a free virus particle, $2a$ the long diameter of an immobilised virus particle and $2c$ the short diameter of an immobilised virus particle.
Where $r$, $a$ and $c$ are as defined in Figure 6.12. The collapse was assumed to be symmetric laterally (i.e. $b = a$). Thus the width of the immobilised virus particle could be shown to be:

$$2a = 2 \sqrt{(r^2/c)}$$

The effect of an ellipsoidal collapse on the virus particles was calculated using the published dimensions of the virus particles, the AFM measured particle heights and the above equation. In each case the AFM measured widths exceeded the calculated widths (Table 6.3). This indicated that the virus particles, if particle volume was conserved, were not collapsing into an ellipsoidal shape but into structures that had a narrow upper region and a relatively broad base.
### Table 6.3

The effect of an ellipsoidal collapse on the virus particles was calculated using the published dimensions of the virus particles, the AFM measured height dimensions and the equation $2a = 2 \sqrt{r^2/c}$. In each case the measured widths of the virus particles exceed the calculated widths. **NOTE:** All values shown are in nanometers (nm).

<table>
<thead>
<tr>
<th>Virus</th>
<th>Radius of virus (r)</th>
<th>Measured height (2c)</th>
<th>Half measured height (c)</th>
<th>Calculated width (2a)</th>
<th>Measured width</th>
</tr>
</thead>
<tbody>
<tr>
<td>DA (anti-TMEV)</td>
<td>24-30</td>
<td>15</td>
<td>7.5</td>
<td>85-120</td>
<td>446</td>
</tr>
<tr>
<td>L10 capsids (anti-A5)</td>
<td>23</td>
<td>13</td>
<td>6.5</td>
<td>87</td>
<td>218</td>
</tr>
<tr>
<td>L10 enveloped (anti-E1)</td>
<td>32</td>
<td>28</td>
<td>14</td>
<td>97</td>
<td>160</td>
</tr>
<tr>
<td>L10 enveloped (anti-E2)</td>
<td>32</td>
<td>36</td>
<td>18</td>
<td>85</td>
<td>164</td>
</tr>
</tbody>
</table>
6.4: Conclusions.

The use of antibody-coated wafers has been proven to be an effective method for the immobilisation of virus particles. This method proved suitable for both enveloped and non-enveloped viruses. It is envisaged that this technique is applicable for any antigenic structure once a suitable antibody is available. Virus particles were successfully imaged and viral dimensions determined. In all cases the virus particles appear to collapse when they are immobilised with the viral heights typically reducing from $\frac{1}{3}$ - $\frac{1}{2}$ of the actual diameters. The observed decrease in particle height coincides with an increase in particle width which is consistent with the particles collapsing upon immobilisation.
Conclusions and Future Directions
Conclusions:

- The primary objective of this study was to observe (using both enveloped and non-enveloped viruses) viral entry into and egress from cultured cells using the AFM. An essential step in achieving this objective was the optimisation of cell sample preparation for AFM examination. Results of optimisation experiments revealed that cultured cells seeded onto glass coverslips, at a density which ensured sub-confluency at the time of imaging, and fixed with 4% paraformaldehyde were suitable for AFM examination. AFM images of BHK-21 cells revealed fibroblastic cell morphologies while AT₃ cells exhibited globular cell morphologies.

- CPE, TUNEL and immunocytochemistry studies revealed that TMEV's were unable to produce progeny virus in AT₃ cells. This may be explained by either an inability of the virus to enter these cell lines or an inability, once entry has been achieved, of the virus to replicate within the cell. AFM analysis of viral entry into cultured cells demonstrated that TMEV and SFV-infected AT₃Neo cells were smoother than the mock-infected control cells. This data suggests that TMEV are capable of gaining entry into AT₃Neo cells and that CPE is not produced by TMEV in these cells due to an inability of the viruses to replicate.
AFM analysis of the egress of progeny viruses from cultured cells demonstrated that cytopathic deterioration of the cells can be visualised. The surfaces of virally infected cells at 75% CPE were rougher than those of mock-infected control cells. Furthermore, the cell heights of virally-infected cultured cells were increased in comparison to the mock-infected control cells. It is postulated that these effects may be due to the presence of progeny virus aggregates on infected cell surfaces.

Viscoelasticity analysis of viral entry into cultured cells demonstrated no consistent effect on cell viscoelasticity, with certain virus strains causing softening of infected cell lines while others resulted in cell stiffening. Viscoelasticity analysis also revealed that enveloped and non-enveloped viruses do not have comparable/contrasting effects on relative cell viscoelasticity at the stage of viral entry.

Viscoelasticity analysis of progeny viral emergence from cultured cells also demonstrated no consistent effect on cell viscoelasticity. Analysis of the emergence of enveloped progeny virus did reveal, in certain cases, that virally-infected cultured cells were stiffer than mock-infected control cells. This effect may reflect the emergence strategy of enveloped viruses in that the cell membrane is being removed by the egressing virus thereby causing an increase in cell volume: cell surface ratio which may be resulting in the cells becoming more compact and firm.
The AFM analysis of virus particles demonstrated that antibody-coated wafers are an effective means for the immobilisation of both enveloped and non-enveloped virus particles. Virus particles were successfully imaged and in all cases the virus particles appear to collapse when they are immobilised.
Future directions:

- AFM analysis of virally-infected cultured cells has numerous applications in the field of virology as countless types of viruses can be examined once a suitable cell line exists. The application of the AFM to observe enveloped and non-enveloped viral entry into, and egress from, cultured cells using the AFM may benefit from future experimentation utilising viral labels, such as gold labeled antibodies, which could aid in the direct localisation of virus particles entering into or egressing from cultured cells.

- Viscoelasticity analysis of virally-infected cells also has endless applications in the field of virology. This technique could be particularly applicable to viral infections that result in the rearrangement of the cell cytoskeleton and therefore cause significant changes in cell viscoelasticity. AFM viscoelasticity analysis could also be applicable to the area of cell biology. Potential differences in cell viscoelasticity between normal and abnormal (e.g. cancer) cells could be investigated. In addition, viscoelasticity analysis could be used to explore the effects of chemicals and pharmaceuticals on cells.

- AFM analysis of virus particles offers the prospect of studying the shape and characteristics of the virus particle. A second application of this technique would be the investigation of the effects of anti-viral drugs, chemicals and pharmaceuticals on viruses. By attaching the chemical or pharmaceutical to
the AFM tip, adhesion events between the coated tip and the virus particle could be detected, using the force measurement software of the AFM. This area could also be developed to provide a virus identification system in which multi-antibody coated wafers are employed to isolate the antibodies' corresponding virus.

➢ The AFM could also be applied to the field of immunology. Topographically, it could be used to investigate cell-cell interactions, e.g. between T helper cells and antigen presenting cells. Also, by coating the AFM tip with antibody, the AFM force measurement capabilities could be used to examine antibody-cell and antibody-antigen interactions.
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Appendix A

Materials
Bovine serum albumin (BSA):

3 g BSA was dissolved in 30 ml sdH2O, 0.2 μm filter sterilised, aliquoted and stored at -20°C.

Cell lines and corresponding media:

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Medium</th>
<th>Supplements</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHK-21</td>
<td>GMEM</td>
<td>NCS, TPB, GPS</td>
</tr>
<tr>
<td>AT₃Neo and AT₃Bcl₂</td>
<td>RPMI 1640</td>
<td>FCS, PS, dexamethasone, G-418</td>
</tr>
</tbody>
</table>

DEAE-dextran:

0.3 g of DEAE-dextran was dissolved in 20 ml of sdH2O, 0.2 μm filter sterilised, aliquoted and stored at 4°C.

5 % Difco noble agar:

50 g of Difco noble agar (Difco Laboratories, Michigan, USA) was dissolved in 1 litre of dH2O, aliquoted and autoclaved before use.

Formal saline:

85 g of NaCl was dissolved in 9 litres of dH2O and 1 litre of formaldehyde (37 % solution) was added.
G-418 (geneticin) 100 mg/ml:

135 mg of G-418 (microbial potency 740 µg/mg) was dissolved in 1 ml of sdH2O, filter sterilised and aliquoted before use. The G-418 was stored at -20°C until required.

20 % Goat serum:

2 ml Goat serum was added to 8 ml of TBS.

0.3 % H2O2:

1 ml of H2O2 (30 % w/v) was added to 99 ml of TBS. The H2O2 was prepared freshly prior to use.

LB broth:

20 g of LB broth powder was dissolved in 1 litre of dH2O, aliquoted and autoclaved before use.

MgCl2:

8 g of MgCl2 was dissolved in 100 ml of sdH2O, 0.2 µm filter sterilised and stored at 4°C.
1x PBS:

Part A: Stock Solution 10x
1.37 mM NaCl, 268 mM KCl, 81 mM Na$_2$HPO$_4$ and 14 mM KH$_2$PO$_4$. pH was adjusted to 6.7 and the solution was autoclaved before use.

Part B: Working solution 1x
10x stock was diluted 1 in 10 with sdH$_2$O.

Toluidine blue:
2.5 g of Toluidine blue was dissolved in 2.5 litres of tap H$_2$O.

0.3 % Triton X-100 in TBS:
3 ml of Triton X-100 was added to 100ml of TBS.

Trypsin:
2 ml trypsin was added to 18 ml of 1x PBS.

Tryptose phosphate broth (TPB):
39.5 g of tryptose phosphate powder was dissolved in 1 litre of dH$_2$O, aliquoted and autoclaved before use. The TPB was stored at 4°C until required.
Appendix B

Cell culture experimental methods
Sterility testing.

Approximately 2 ml of test reagent and approximately 10 ml of LB broth were added to a sterile universal container. The container was incubated in a 37°C incubator. The container was examined for any evidence of bacterial growth over the following two days.

Passing cultured cells.

A flask of cells was examined for confluence using the inverted light microscope (TMS-F, Nikon, Japan). The medium was decanted from the flask into a clean container, taking care not to splash any medium into the flask of cells. The monolayer was rinsed twice with 1x PBS. Trypsin (approximately 3 ml for a 1 x T75 cm² flask) was added and the flask was rocked gently to spread the trypsin evenly over the monolayer. As soon as the monolayer had detached, approximately 7 ml of medium was added to the flask and the cells were aspirated gently to break up clumps. The cell suspension was added to a sterile 50 ml Falcon tube and centrifuged at approximately 1,800 g for 5 min to pellet the cells. The supernatant was decanted, the tube was gently tapped to dislodge the pellet, 10 ml of medium was added and the cells were gently aspirated until a homogenous suspension was obtained. The cells were counted if required and a new flask was seeded with the desired amount of cells. Approximately 15 ml of medium was added to a 1 x T75 cm² flask. The flask was labeled, dated and incubated at 37°C, 5% CO₂ until the required degree of confluence was reached.
Counting cells.

50 µl of cell suspension was added to 500 µl of Trypan blue. The cells were counted using a Neubaeur counting chamber. The number of cells in the cell suspension was calculated using the following formula:

No. of cells counted per 16 large squares x dilution factor x 10⁴ = no. of cells/ml

Virus plaque assay.

Part A - Setting up plates.

A flask of BHK-21 cells was examined for confluence using the inverted light microscope (TMS-F, Nikon, Japan). The cells were passed to obtain a cell suspension and a cell count was performed on the cell suspension. The amount of cell suspension required to set up 10 mm x 35mm plates was calculated. Each plate was seeded with approximately 1 x 10⁶ cells, in 3 ml of BHK-21 medium and incubated 37°C, 5% CO₂. The cells were semi-confluent 24 h later and a virus assay was then performed.

Part B - Virus Assay.

The virus was diluted in 1x PBS. Serial dilutions of 1:100 were prepared; to achieve this 5 µl of virus was added to 495 µl of PBS. This gave dilutions as follows N, 10⁻², 10⁻⁴, 10⁻⁶ etc. 5% Difco agar was melted and transferred to a 56°C water bath until required. The medium was decanted from the 10 mm x 35 mm plates into a clean container, care was taken not to splash any medium into the cells and the cells were rinsed twice with 1x PBS. 100 µl of the appropriate
virus dilution was added to each plate, in duplicate and allowed to absorb at room temperature for approximately 45 min. 2 control plates were "mock-infected" with 1x PBS. The plates were rocked gently every 15 min. The agar was cooled and diluted to 0.05% with warm PAM. Approximately 1.5 ml of agar medium was added to each plate and allowed to set at room temperature. Each plate was gently flooded with PAM and incubated at 37°C, 5% CO₂ for 2 days (until plaques were visible).

Part C - Fixation and staining.

The agar was decanted from the plates into a suitable container. The cells were fixed with formal saline for a minimum of 10 min. The formal saline was decanted and the cells were stained with Toluidine Blue for approximately 10 min. The Toluidine Blue was decanted, the plates were rinsed with tap water, dried and the plaques were counted.

Freezing cells.

A flask of BHK-21 cells was examined for confluence using the inverted light microscope (TMS-F, Nikon, Japan). The cells were passed until the stage of centrifugation. The cell suspension was centrifuged at approximately 1,800 g for 10 min. The supernatant was decanted, the tube was gently tapped to dislodge the pellet, 1 ml of cold freezing medium (1 in 10 dilution of DMSO in NCS) was added and the cells were gently aspirated until a homogenous suspension was obtained. The cell suspension was transferred into a freezing vial, frozen at-80°C
and transferred to a -150°C cryogenic freezer. The remaining cell suspension was sterility tested.

**Thawing cells.**

The cells were removed from the freezer and thawed in a 37°C water bath. The thawed cells were added to 10 ml of medium and centrifuged at 1090 rpm for 5 min (this served to remove the freezing medium). The supernatant was decanted and the tube was gently tapped to dislodge the pellet of cells. 5 ml of medium was added and the cells were gently aspirated until a homogenous suspension was obtained. The cell suspension and 10 ml of extra medium were added to a 1 x T 75 cm² flask and incubated overnight at 37°C, 5% CO₂.
Appendix C

Calculations for virus particle analysis
The following is an example of the calculations used to determine the number of virus particles required to form a monolayer on a silicon wafer.

**Area of the virus particle:**

SFV particles are 70 nm in diameter, by multiplication of length by breadth the area of an SFV virus particle can be determined.

Area of virus particle 70 nm x 70 nm = 4900 nm$^2$

**Area of the silicon wafer:**

Also calculated by multiplication of length by breadth, the diameter of the silicon wafers used was 4 mm.

Area of wafer 4 mm x 4 mm = 16 mm$^2$

**Number of virus particles required:**

The number of virus particles required to form a monolayer on the silicon wafer surface is calculated by dividing the area of the wafer by the area of the virus particle.

\[
\text{Number of virus particles required} = \frac{16 \text{ mm}^2}{4900 \text{ nm}^2} = \frac{16 \times 10^{-6} \text{ m}^2}{4.9 \times 10^{-15} \text{ m}^2} = 3.27 \times 10^9
\]
Surface volume of silicon wafer:

The determination of the maximum volume of liquid which can be applied to the silicon wafer surface was calculated using the formula for the computation of the volume of a hemisphere.

\[
\text{Volume of a hemisphere} = \frac{2}{3} \pi r^3
\]
\[
= \frac{2}{3} \times 3.14 \times (2 \text{ mm})^3
\]
\[
= 16.7 \text{ mm}^3
\]

\[
1 \text{ mm}^3 = 1 \mu l
\]
\[
= 16.7 \mu l
\]

Therefore from the above calculations, to obtain a monolayer of SFV particles on a 4 mm x 4 mm silicon wafer, \(3.27 \times 10^9\) virus particles in 16.7 \(\mu\)l are required.