1   How have retrovirus pseudotypes contributed to our understanding of viral entry?

2

3   **Abstract**

4   Study of virus entry into host cells is important for understanding viral tropism and
5   pathogenesis. Studying the entry of in vitro cultured viruses is not always practicable. Study
6   of highly pathogenic viruses, viruses that do not grow in culture, and viruses that rapidly
7   change phenotype in vitro can all benefit from alternative models of entry. Retrovirus
8   particles can be engineered to display the envelope proteins of heterologous enveloped
9   viruses. This approach, broadly termed 'pseudotyping', is an important technique for
10   interrogating virus entry. In this perspective we consider how retrovirus pseudotypes have
11   addressed these challenges and improved our understanding of the entry pathways of
12   diverse virus species, including Ebolavirus, human immunodeficiency virus and hepatitis C
13   virus.

14   **Keywords:**

15   Pseudotype

16   Pseudoparticle

17   Entry pathway

18

19   Executive Summary

20   • The entry of a virion into a host cell is an essential step in viral life cycles. As such, entry is a
21   potential target for clinical intervention. Viral entry pathways are also the target for
22   neutralizing antibodies generated by immunization with vaccines possessing virion
23   components.
24   • Enveloped viruses cause a range of diseases in humans and animals, from acute infections
25   such as Ebola and Influenza, to chronic infections such as hepatitis B virus, hepatitis C virus
26   and human immunodeficiency virus. The entry pathways of these enveloped viruses are
27   complex and vary between virus species. Greater understanding of the steps of cell binding,
28   internalisation and fusion are important for designing novel therapies for virus infections.
29   • The ability to experimentally generate retroviruses pseudotyped with heterologous
30   envelope proteins provides a flexible platform for investigating the entry pathways of a wide
31   range of genetically diverse viruses. While culture models exist for some viruses that permit
32   investigation of entry there are examples of viruses where investigating entry in isolation is
33   beneficial.
34   • Entry of highly pathogenic viruses such as Ebolavirus and Rabies virus can be studied using
35   retroviral pseudotypes as a surrogate entry model, without the safety concerns associated
36   with working with full-length infectious viral genomes
37   • In vitro culture of viruses with RNA genomes can lead to culture-associated adaptation and
38   perturbation of the phenotype of the virus. The use of retroviral pseudotypes to study the
39   entry pathways of these viruses can address this problem, using high-fidelity PCR to
40   generate accurate representations of the envelope protein sequences from clinical
41   specimens.
42   • Retroviral pseudotypes can be used to study the entry pathways of viruses that do not
43   readily replicate in culture, such as hepatitis C virus.
44   • For those viruses that encode proteins in different overlapping open reading frames, the use
45   of retroviral pseudotypes can facilitate specific investigation of the entry phenotype of
46   mutants in isolation from the effects of coding changes in other virus-encoded proteins.
47   • Retroviral pseudotypes can also be used to identify host factors that act as restriction factors
48   that act on the entry pathways of diverse viral species.

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Introduction

Viruses possessing host-derived envelope membranes make up a large number of viral human pathogens, including members of the positive-stranded and negative stranded RNA viruses. The on-going challenges of global eradication of chronic virus infections such as human immunodeficiency virus 1 (HIV-1), hepatitis B virus (HBV) and hepatitis C virus (HCV) demonstrate that new therapeutic approaches are required for these infections. In addition, recent zoonotic outbreaks such as new influenza virus variants, Ebola virus and Zika virus have highlighted the need for improved surveillance and greater understanding of the pathogenicity of emerging viruses. Enveloped viruses important to human health are genetically diverse, for example members of the families *Flaviviridae*, *Retroviridae*, *Filoviridae*, *Arenaviridae*, *Orthomyxoviridae* and *Coronaviridae*. These virus families have distinct replication strategies and present unique challenges for therapy. However, expression of a virus-encoded trans-membrane protein that mediates the entry process is a common feature of all these virus species. As such, greater understanding of these distinct entry pathways may inform development of novel interventions for virus infections.

Entry pathways of enveloped viruses

Understanding virus entry pathways is important for determining host range and tissue specificity of different virus infections. Enveloped viruses gain entry to a host cell by one of two main mechanisms, both of which require attachment of a virus particle to host-encoded receptors on the plasma membrane (reviewed in [1]). Viruses can utilise the host endocytosis pathway to internalise particles into endosomes, leading to pH-dependent fusion of the viral envelope with the host cell membrane in early endosomes (e.g. Nipah virus [2]) or at lower pH after endosome fusion with lysosomes (e.g. Dengue virus [3]). Alternatively, viral fusion and entry can take place at the cell surface in a pH-independent manner, with receptor engagement triggering membrane rearrangement to result in internalisation of the viral nucleocapsid at the plasma membrane. HIV-1 has long been cited as an example of pH-independent plasma membrane fusion [4, 5]. More recent analyses have demonstrated that HIV-1 fusion occurs in endosomes [6], although this has been disputed [7]. Irrespective of the mechanism of entry, virus-encoded membrane-spanning proteins mediate these receptor binding and membrane fusion events, resulting in internalisation of the capsid into the cell cytoplasm. As such, the envelope proteins are the main target of neutralizing antibodies, which block receptor interactions and aggregate virus particles. In addition, envelope proteins are often highly glycosylated, which may help protect the virus from host immune recognition [8]. Intimate knowledge of entry pathways can inform development of new therapies for virus infections, and has already led to the development of clinically approved drugs targeting entry of HIV-1 [9] and respiratory syncytial virus (RSV) [10].

It is important to note that the entry pathways of non-enveloped viruses is fundamentally different to that of enveloped viruses. Entry of these viruses does not involve membrane fusion and requires penetration of the virus particle through a host cell membrane (reviewed in [11]). This penetration is mediated by the virus' outer capsid antigens, which typically form highly ordered, symmetrical structures that contribute to virion structure. A

94 good example of this is the Bluetongue virus (BTV) particle, which is formed of concentric
95 layers of VP3, VP7 and VP5, with the outer-most virus protein VP2 embedded into this
96 layered structure [12]. The surface proteins of enveloped and non-enveloped viruses may
97 share some biochemical characteristics, but the characteristic trans-membrane domain of
98 enveloped-virus surface proteins is lacking in these viruses.

99 While laboratory investigation of the entry pathways of many viruses can be performed
100 using cultured virus isolates, there are situations where this is not desirable: 1) Highly
101 pathogenic viruses that require high level containment facilities; 2) Viruses that rapidly
102 accumulate mutations and culture adaptations, and where investigation of accurate
103 representations of virus populations is important; 3) Viruses that do not readily grow in
104 culture, or where host restriction in cultured cells occurs at a post-entry step; 4) Viruses for
105 which molecular clones are not available, or where reverse-genetics approaches are desired
106 to manipulate the glycoproteins to investigate molecular determinants of entry pathways.
107 This latter group includes viruses with overlapping reading frames, where mutations in the
108 envelope proteins results in additional amino acid substitutions in other viral proteins. One
109 possible approach to overcome the limitations of virus culture is the use of chimeric viruses.
110 Because the envelope proteins are necessary and sufficient for entry, it is possible to study
111 entry pathways using experimental models that reconstitute only the viral envelope protein
112 binding to cell surfaces. This has led to the development of a range of experimental models
113 with which to study viral tropism and entry, including infection with virus-like particles [13,
114 14], infectious pseudotypes [15-17], protein binding assays [18, 19] and liposome fusion
115 assays [20, 21]. These models also facilitate assessment of inhibitors that specifically target
116 viral entry pathways in isolation from other steps of the replication cycle [22, 23].

117

118 **Retrovirus-based pseudotypes**

119 Retroviruses are excellent platforms for creating chimeric virus particles with which to
120 investigate viral entry. In contrast to many viruses, retroviruses are able to incorporate
121 foreign proteins into their envelope membrane, including host-derived proteins and
122 envelope proteins of other viruses [24-29]. This promiscuous protein incorporation naturally
123 results in phenotype mixing in cells infected with different species of retrovirus [30, 31]. The
124 wide range of viral surface proteins that have been successfully incorporated into
125 retroviruses has recently been reviewed [32]. Following infection retroviruses deliver and
126 integrate a DNA copy of their RNA genome into the host genome of an infected cell during
127 replication. This facilitates introduction of reporter genes into retroviral genomes along with
128 strong promoters, resulting in expression of the integrated reporter genes. This permits
129 design of rapid, sensitive quantitative infection assays that can be easily re-purposed for
130 studying the entry of a range of enveloped viruses (Figure 1). It should be noted that while
131 we consider only retrovirus pseudotypes in this Perspective, heterologous viral
132 glycoproteins have been successfully pseudotyped onto vesicular stomatitis virus (VSV)
133 particles [33]. VSV pseudotypes, along with approaches using enveloped virus-like particles
134 (VLPs) [34] have also made a significant contribution to our understanding of viral entry
135 pathways. These experimental models are restricted to studying the surface proteins of

136 enveloped viruses, as the surface capsid proteins of nonenveloped viruses cannot be readily
137 incorporated into the chimeric retrovirus particles as they lack a trans-membrane domain.
138 Retroviral pseudotyping provides an experimental model to investigate the early stages of
139 entry of enveloped viruses without introducing sequence adaptations associated with *in*
140 *vitro* culture and passage of viruses. In addition, this experimental system provides a robust
141 model with which to rapidly investigate the phenotype of envelope proteins representing
142 diverse viral variants. It is important to distinguish between pseudotypes generated with the
143 two main groups of retrovirus packaging constructs: lentiviruses and gammaretroviruses.
144 Pseudotypes based on lentiviruses such HIV-1 and simian immunodeficiency virus (SIV) are
145 able to infect and integrate into terminally-differentiated cells. In contrast, those based on
146 gammaretroviruses such as murine leukaemia virus (MLV) and gibbon ape leukaemia virus
147 (GALV) generally require nuclear membrane degradation during mitosis to deliver and
148 integrate their genome into that of the host cell, limiting investigations of virus entry
149 pathways to actively dividing cells [35]. However, pseudotypes of the gammaretrovirus
150 Friend MLV (F-MLV) may transduce non-dividing cells under certain conditions [36]. Despite
151 these differences both HIV-1 and MLV have been popular choices for pseudotype virus
152 production, with optimised protocols for both species [37, 38].

153 Retroviral pseudotyping was originally applied to investigations into retrovirus entry using
154 surface proteins encoded by the *env* gene (surface glycoprotein; SU and transmembrane
155 protein; TM) [25, 27]. These retroviruses were also found to incorporate heterologous viral
156 surface proteins [28, 39], permitting analysis of a wide range of variants [24]. Glycoproteins
157 recovered from a range of retrovirus species have been pseudotyped onto heterotypic
158 retrovirus backbones, including gammaretroviruses (ecotropic and amphotropic MLV and
159 GALV), and lentiviruses. These studies revealed that different retrovirus species can possess
160 pH-dependent or pH-independent entry pathways [40, 41], suggesting that different
161 retroviruses use distinct entry pathways.

162 The methodological approaches for the generating retroviral pseudotypes for studies of
163 virus entry have been described and reviewed many times [42-44]. Here we discuss how
164 retrovirus pseudotypes have contributed to the understanding of receptor usage, the cell
165 biology of viral entry, and the consequences of genetic diversity on envelope protein
166 function.

167 **Application of retroviral pseudotypes to investigating entry of highly pathogenic viruses**

168 Filovirus infections, including Ebolavirus, are a major cause of haemorrhagic fevers and are
169 increasingly studied after the unprecedented 2013-16 outbreak in West Africa. The entry
170 pathways of haemorrhagic filoviruses are important targets for intervention, with a cocktail
171 of therapeutic antibodies (Zmapp) being successfully used to protect against infection [45].
172 While Ebolavirus (EBOV) and Marburg virus (MARV) have demonstrated the ability to rapidly
173 emerge in resource-limited settings, other members of this family result in lower
174 pathogenicity infections in humans. Their highly pathogenic nature makes investigations of
175 Ebolaviruses restricted to containment level 4 laboratories. As such, retroviral pseudotypes
176 provide a safe, flexible platform for investigating the entry pathways of these viruses.
177 Filoviruses express two glycoproteins, GP_1 and GP_2, cleaved from a single precursor [46],

178 which mediate receptor binding and fusion, respectively. Retroviruses pseudotyped with the
179 EBOV/MARV glycoproteins have been used to investigate the entry pathway in great detail
180 [15, 16]. The folate receptor α was initially proposed as a co-factor in the entry pathways of
181 both viruses [47], but this has subsequently been queried [48]. T cell immunoglobulin and
182 mucin domain 1 (TIM-1) was more recently identified as an entry factor for both viruses by
183 assessing EBOV pseudotype entry in a panel of transformed cell lines with defined gene
184 expression [49]. A similar approach identified members of the Tyro3 transmembrane
185 tyrosine kinases (Axl, Dtk and Mer) as entry cofactors [50]. Differences in entry of EBOV and
186 MARV were also revealed using pseudotypes. MARV pseudotype entry was resistant to
187 glycosylation inhibitors in target cells, in contrast to the tunicamycin- and Endoglycosidase
188 H-sensitive entry of EBOV [51]. This highlighted differences in cellular receptors for these
189 two related viruses. Retrovirus pseudotypes have recently been applied to identifying the
190 Niemann-Pick C1 (NPC1) protein as the major GP-binding receptor for Ebolavirus [52, 53].
191 They have also been used to identify molecular determinants of receptor tropism in
192 different strains [54]. Together, these studies demonstrate that retrovirus pseudotypes are
193 a powerful tool for investigating the receptor-mediated entry pathway of a range of
194 filoviruses, which would otherwise require high-containment laboratories. These models
195 have accelerated Ebolavirus research and recently assisted with defining the mode of action
196 of antiviral antibodies that target the entry pathway [55].

197 Rhabdovirus glycoproteins, including those of rabies virus, have been readily pseudotyped
198 onto retrovirus backbones, including infectious equine anaemia virus and HIV-1 [56, 57].
199 These pseudotypes have been used to investigate the neuronal transport of virions
200 mediated by the rabies virus glycoprotein [58]. Retrovirus pseudotypes of rabies, VSV and
201 mokola viruses (as well as EBOV and lymphocytic choriomeningitis virus – LCMV) enabled
202 investigation of neuronal cell tropism *in vivo*. Injection of these pseudotypes into the brains
203 of mice facilitated post-mortem identification of permissive cell types by staining for the
204 beta-galactosidase reporter enzyme packaged by the pseudotypes [59]. A similar technique
205 has been used for skin cell tropism of VSV [60]. Furthermore, lentivirus pseudotyping of
206 chimeric glycoproteins comprising domains of rabies G and VSV G enabled phenotyping of
207 fusion glycoproteins possessing unique entry characteristics and neuronal cell tropism for
208 use in retrograde transduction systems [61].

209

210 **Applications with highly diverse virus populations**

211 An important use of retrovirus pseudotypes is accurate assessment of the phenotypes of
212 envelope proteins recovered from viruses that undergo rapid mutation and selection *in vivo*.
213 Passaging these viruses in cell culture can often result in rapid adaptation to culture, as the
214 absence of the constraining environment of the natural host cell and immune responses
215 alters the selection pressures acting on a viral quasispecies. Isolating viral envelope protein
216 gene sequences with high-fidelity approaches allows accurate assessment of the properties
217 of circulating variants with pseudotypes [62, 63]. Studies of HIV-1 entry provide a good
218 example of this approach. The error-prone nature of the virus-encoded reverse
219 transcriptase facilitates rapid adaptation in tissue culture with mutations in the envelope

220 glycoprotein gene *env* resulting in phenotypic changes [64, 65]. This hindered research into
221 HIV entry, as receptor usage of laboratory-adapted, cultured strains was inconsistent with
222 those naturally infecting human hosts. Retroviruses pseudotyped with HIV-1 envelope
223 glycoproteins were used to identify host receptors as determinants of entry. HIV variants
224 have differential usage of CD4 and the chemokine receptors CXCR4 and CCR5 [66-68], and it
225 was recently demonstrated that CD4 usage by HIV-1 pseudotypes is linked to breadth of
226 tropism [69].

227 HIV-1 constructs pseudotyped with envelope glycoproteins that mediate pH-independent
228 entry were also demonstrated to be enhanced by the action of the HIV-1 nef protein,
229 revealing a role for nef in entry [70]. The entry properties of genetically diverse HIV-1 *env*
230 clones were assessed using pseudotyped virus infection of peripheral blood mononuclear
231 cells (PBMCs) [71]. The development of rapid HIV-1 pseudotype screening assays [72] has
232 more recently facilitated assessment of entry of genetically diverse strains of HIV-1 for
233 studies of entry inhibition [73].

234 **Applications to viruses that do not grow in culture**

235 Study of the hepatitis C virus (HCV) entry pathway presents additional challenges to that of
236 HIV-1, as HCV is not readily propagated *in vitro*. Only one strain of HCV (JFH-1) robustly
237 replicates in tissue culture without the requirement for culture adaptations. Laboratory-
238 generated chimeric viruses based on JFH-1 rapidly accumulate *in vitro* adaptations [74]. The
239 error-prone replication of HCV coupled with the persistent, chronic nature of infection
240 results in extreme levels of diversity between isolates identified in different host
241 backgrounds [75]. The diversity observed between different HCV genotypes far exceeds that
242 observed for other chronic infections, such as HIV-1 [76]. HCV encodes two envelope
243 proteins, E1 and E2, which are necessary and sufficient for mediating entry of the virus.
244 However, these genes are able to tolerate extreme genetic diversity (reviewed in [75]), and
245 are common sites of *in vitro* culture adaptations. Nucleotide sequence variation approaches
246 40% between individual viruses. Early studies of the entry pathway of HCV were severely
247 limited by the lack of robust cell culture models in transformed hepatoma cultures. The
248 introduction of retroviruses pseudotyped with the HCV E1 and E2 proteins (hepatitis C virus
249 pseudoparticles; HCVpp) facilitated rapid developments in our understanding of the
250 complex receptor cascade required for HCV entry (see Figure 1) [77, 78]. These studies
251 initially identified CD81 and SR-BI as key receptors for entry [79], and later identified tight
252 junction proteins including claudins [80, 81] and occludin [82] as key entry factors that play
253 a role in the entry cascade. HCVpp were also used to identify the sequential order of events
254 in the entry cascade [83-85]. E2 is the major receptor binding protein, while E1 plays a role
255 in chaperoning the folding of the E2 protein and contributes to envelope membrane fusion
256 [86]. HCVpp were used to map the interactions between these glycoproteins and receptors
257 to the amino acid level by studies using panels of individual point-mutation variants in
258 controlled genetic backgrounds [87-90]. The fusion mechanism for HCV is yet to be
259 completely resolved, but studies have located elements in both the E1 and E2 proteins that
260 contribute to fusion [86, 91]. More recently, mutations that affect the pH-dependent nature

261 of HCV entry have been tested using pseudotypes, implicating the hypervariable 1 (HVR-1)
262 in pH-dependent fusion [92].

263 HCV tissue tropism has been investigated with HCVpp. These particles have been found to
264 enter neuroblastoma cell lines [93] and neuroepithelioma cells [94]. Pseudotypes were also
265 used to analyse the contribution of kinases in the HCV entry pathway [95], the process of
266 endocytosis in the entry cascade [96] and the importance of cell polarity in HCV receptor
267 association [97]. The discovery that human serum enhances infection of HCVpp into
268 hepatocytes led to investigations of lipoprotein-mediated HCV infection. High-density
269 lipoprotein (HDL) was discovered to enhance infection by accelerating endocytosis [98],
270 through interactions with the SR-BI receptor [99] and the HVR-1 region of the HCV E2
271 glycoprotein [100].

272 The use of HCVpp to interrogate the properties of genetically diverse viral isolates has
273 revealed that while all genotypes of HCV use conserved entry pathways [101], entry
274 phenotypes vary considerably between isolates [102, 103]. Closely related members of an
275 individual quasispecies can have significantly different entry efficiencies into host cells [38,
276 104]. These entry assays revealed different phenotypes of variants that are preferentially
277 transmitted between hosts [63]. By comparing E1/E2 nucleotide sequences of genetically
278 diverse functional HCVpp, discontinuous conserved receptor binding regions were proposed
279 [87]. Interrogation of these regions with point mutations identified critical residues involved
280 in the interaction with CD81. The success of this approach was revealed when core crystal
281 structures of the E2 protein became available [105, 106], confirming the discontinuous
282 nature of the conserved amino acids constituting the CD81 binding site.

283 **Application to viruses with overlapping reading frames.**

284 The hepatitis B virus (HBV) life cycle has been studied in detail. However, the entry cascade
285 has only recently been characterised at a molecular level [107]. HBV possesses a variable
286 genome, with 8-10% difference observed on the nucleotide level between strains. HBV is
287 categorised into at least 8 distinct genetic types, which differ in geographic distribution and
288 pathogenicity [108]. The HBV envelope protein (HBV surface antigen - HBsAg) is expressed
289 in three forms, with a common C-terminus but three separate initiation sites that encode
290 the large (L), medium (M) and small (S) forms of the protein. The major sites of variability
291 are in the surface antigen, particularly the antigenic region present in an external loop near
292 the C-terminus. This provides the first challenge to investigating HBV entry, as culture
293 models for rapidly assessing the phenotype of naturally occurring variants are not available
294 [109]. In addition, the HBV genome possesses overlapping coding regions in all three
295 translation frames. Mutations in the surface antigen can simultaneously introduce
296 important mutations in the viral polymerase. As such, it is difficult to individually phenotype
297 the properties of HBsAg variation in cell culture models. Retrovirus pseudotypes facilitated
298 investigation of the HBV entry pathway [110, 111]. Initial studies identified that HBV
299 infected primary human hepatocytes, but not hepatoma cell lines [110, 111], and illustrated
300 the advantages of using lentivirus constructs for studies in terminally differentiated primary
301 cells [110]. These studies also investigated the contributions of the large and small forms of
302 the HBsAg in entry. With the discovery of the sodium taurocholate co-transporting

303   polypeptide (NTCP) as a major entry factor [112] the models of HBV entry have rapidly
304   advanced. Retroviruses pseudotyped with the HBsAg have recently been used to interrogate
305   the steps in NTCP-mediated HBV entry [17], mapping NTCP binding sites to the N-terminus
306   of the large surface antigen.

Application to identifying restriction factors

308   Retrovirus pseudotypes have been extensively employed for serology screening and analysis
309   of neutralising antibodies [32]. They have also been important for the investigation of host
310   cell restriction factors, such as interferon-induced transmembrane proteins (IFITMs). IFITM
311   proteins were originally identified during siRNA screening for inhibitors of highly-pathogenic
312   avian influenza A virus (IAV) replication. However, IFITMs have also been shown to restrict a
313   range of viruses at a replication-independent stage using retrovirus pseudotypes [113-115].
314   Transduction efficiency of pseudotypes bearing the glycoproteins of filo-, corona-, flavi-,
315   rhabdo- or orthomyxo-viruses were shown to be variously inhibited at an entry or
316   membrane-fusion stage by different IFITM proteins. Upregulation of homologous or
317   orthologous IFITM isoforms using lentivirus transduction demonstrated the likelihood of a
318   shared entry pathway or feature by these enveloped RNA viruses. Retrovirus pseudotyping
319   is uniquely suited allow scrutiny of virus entry in isolation from replication enabling the
320   rapid and direct comparative analysis of how this restriction mechanism effects different
321   viruses.

Limitations to using retroviral pseudotypes.

323   While retroviral pseudotypes have proven to be a valuable tool to interrogate the entry
324   pathways of a wide range of virus species, there are potential problems that must be taken
325   into consideration when using this experimental model. The glycoproteins of some virus
326   species are refractive to incorporation into functional retrovirus pseudotypes, leading to
327   undetectable infection, even when using sensitive reporter assays (unpublished data). Even
328   where viral envelope proteins can be successfully pseudotyped, single amino acid
329   substitutions in the viral glycoprotein can have an impact on the conditions required to
330   generate infectious particles [38]. Attempts to pseudotype flaviviruses such as West Nile
331   virus (WNV) and Zika virus have proven difficult, despite related viruses such as HCV being
332   amenable to pseudotype manufacture. This may be linked to the structural role of the
333   envelope glycoproteins in virus species of the genera such as Flavivirus, while the
334   glycoproteins of hepaciviruses such as HCV do not appear to play such a structural role in
335   virions [116].

336   Envelope glycoproteins can display different phenotypes when present on a retrovirus
337   envelope rather than their natural virion. The neutralization phenotype of HIV-1 strains
338   differs between primary virus isolates and their pseudotyped equivalents [73]. Point
339   mutations in the HCV E1/E2 genes do not always result in the same phenotype when
340   pseudotypes are compared to cell-cultured virus [90, 117]. Also, due to the nature of the
341   producer cell lines, some post-translational modifications might not be accurately modelled
342   by pseudotypes. An example of this is the complex of apolipoproteins that form when HCV
343   is assembled in hepatocytes. Without producing the pseudotyped viruses in hepatocytes

344   these essential modifications, particularly incorporation of Apolipoprotein E, do not occur
345   [118, 119]. This could generate misleading data, especially if these virion components
346   impact on the entry pathway of the virus.

347   When preparing pseudotype entry assays for analysing virus entry pathways, it is important
348   to optimise the assay for the envelope protein being tested (reviewed in [44]). Variables
349   such as the type and source of producer cells, the amount and type of plasmids required to
350   generate infectious particles, and the reporter gene all influence the outcome of infection
351   experiments. Of particular importance is the selection of packaging plasmids. Differences in
352   the assembly of murine leukemia virus and human immunodeficiency virus appear to
353   influence incorporation of heterologous viral envelope proteins, and can determine if
354   infection is successful [38]. The selection of appropriate target cells expressing necessary
355   virus entry molecules also influences the ability to assay infection [78]. Together these
356   considerations can impose practical limitations on assay setup, as glycoproteins from
357   different virus species can behave very differently in these assays. Protocols for generation
358   of particles can impact on the function of the expressed particles, with small changes being
359   sufficient to alter function of a pseudotyped envelope protein [38].

360   Finally, there have been concerns about potential contamination of cell lines used for these
361   experiments with ecotropic retroviruses, which may affect results of infectivity assays with
362   pseudotyped retroviruses. However, at least for HIV-1 infection assays, it has been
363   demonstrated that these contaminants do not affect the results of infection assays [120].

364   **Future applications for retroviral pseudotypes**

365   Pseudotypes have proven to be a robust experimental system to investigate the entry
366   pathways of a wide range of genetically diverse virus species. The current interest in the
367   potential for emerging (and re-emerging) viral pathogens means that retroviral pseudotypes
368   could be deployed rapidly to identify receptor usage and tropism of newly-discovered
369   pathogens. In addition, there is still plenty to learn about the entry pathways of established
370   viral pathogens such as HCV and HBV. Retroviral pseudotypes will continue to be an
371   essential experimental model for these studies.

372   As viral entry has been highlighted as a potential target for clinical intervention in a wide
373   variety of virus infections, the development of broadly-active inhibitors using these entry
374   models may contribute to preparedness for viral epidemics. Retroviral pseudotypes
375   facilitate high-throughput entry inhibitor screening without the requirement to understand
376   the complexities of entry of a specific virus species. Characterising the interactions between
377   entry inhibitors (such as neutralizing antibodies) and viral envelope proteins will also
378   provide useful tools for investigating the molecular biology of virus entry.

379   Retroviral pseudotypes have contributed to our knowledge of the entry pathways of a wide
380   range of viruses. In addition to the examples given here, there are many studies of virus
381   entry that utilise this model, including those of coronaviruses and Influenza viruses. While
382   this Perspective article cannot hope to exhaustively cover each application, we hope that
383   the examples provided here illustrate the versatility of pseudotypes as models of virus
384   entry.

385

386   Figures

387   **Figure 1. Generation of retroviruses pseudotyped with heterologous viral envelope**
388   **proteins. A)** In second-generation pseudotype models, retroviral pseudotypes are produced
389   using genes encoded on three separate plasmids; a packaging vector possessing the entire
390   retroviral gag/pol open reading frame of a specific retrovirus (usually HIV-1 or MLV; blue), a
391   reporter vector possessing a reporter gene (usually either luciferase, green fluorescent
392   protein, or β-galactosidase; green), the 3' and 5' LTRs of a retrovirus matched to the
393   packaging vector, along with a strong promoter and a retrovirus packaging signal (ψ); and a
394   plasmid encoding the glycoprotein(s) of a heterologous virus of interest (yellow/orange).
395   When transfected together into a suitable producer cell line (such as the human embryonic
396   kidney cell line 293T), protein over-expression is driven by the strong CMV immediate-early
397   promoter upstream of each gene. Retroviral particles are produced possessing the desired
398   viral glycoprotein, and are released from the transfected cells into the surrounding media.
399   **B)** In this way the envelope protein-encoding plasmid can be exchanged to produce particles
400   mimicking different virus species.

401   **Figure 2. Retrovirus pseudotypes can be used to reveal many aspects of a virus entry**
402   **cascade.** The entry pathway of hepatitis C virus provides an excellent example of how
403   retroviral pseudotypes can be used to dissect the complex series of events that result in
404   internalisation of a virus into a host cell. **1.** Studies utilising retrovirus pseudotypes were
405   used to identify essential molecular interactions between viral proteins and host cell
406   receptors. CD81 and SR-B1 were identified as key receptors that initiate early events in the
407   entry cascade [79]. **2.** The dynamic nature of interactions between receptor complexes can
408   also be investigated. For HCV, tight junction proteins were identified as co-factors for entry,
409   interacting dynamically with CD81 to traffic virus particles from the cell's apical surface to
410   tight junctions [80, 81, 85]. **3.** Species-specific receptors can be identified. Another tight
411   junction protein, occludin, was found to be species-specific requirement for HCV entry [82].
412   **4.** Pseudotypes can reveal the sequence of events that result in entry of the virus genome
413   into the cell. HCV binding and entry involves sequential recruitment of host cell co-factors
414   [83]. **5.** Host factors other than viral receptors can contribute to virus entry. For HCV,
415   interaction between high density lipoprotein (HDL) and the SR-B1 receptor enhances
416   infectivity [98-100]. **6.** Specific events leading to fusion of virus envelope and host
417   membrane can be interrogated. pH-dependent membrane fusion was found to be mediated
418   by specific regions of the HCV glycoproteins using retroviral pseudotype models [91]. **7.** In
419   addition, specific conserved amino acids were found to be involved in E1-E2 interactions and
420   receptor binding events [87, 88].

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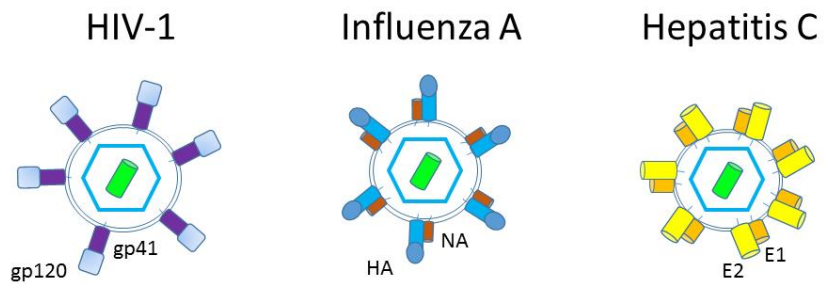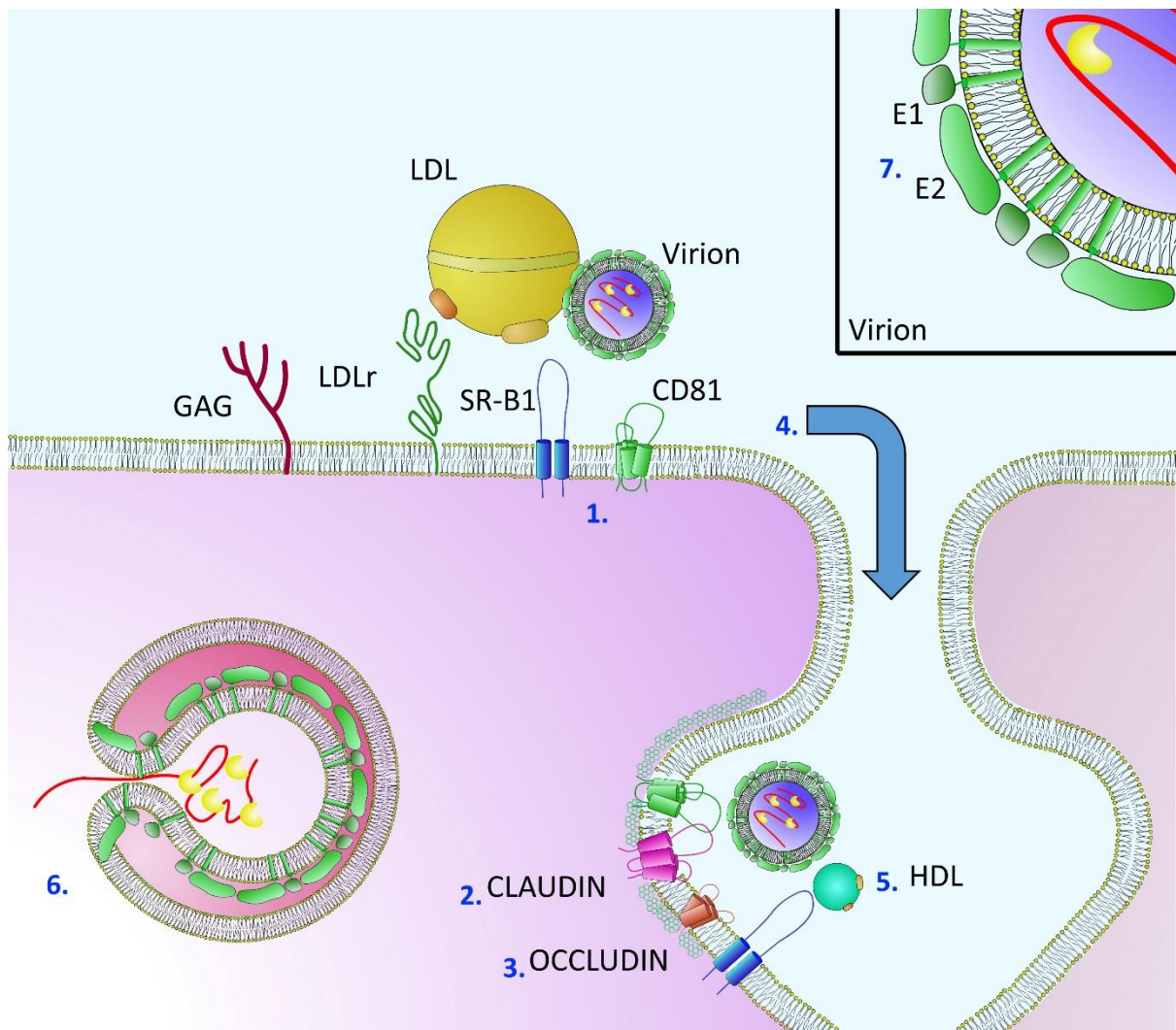721   (2009).

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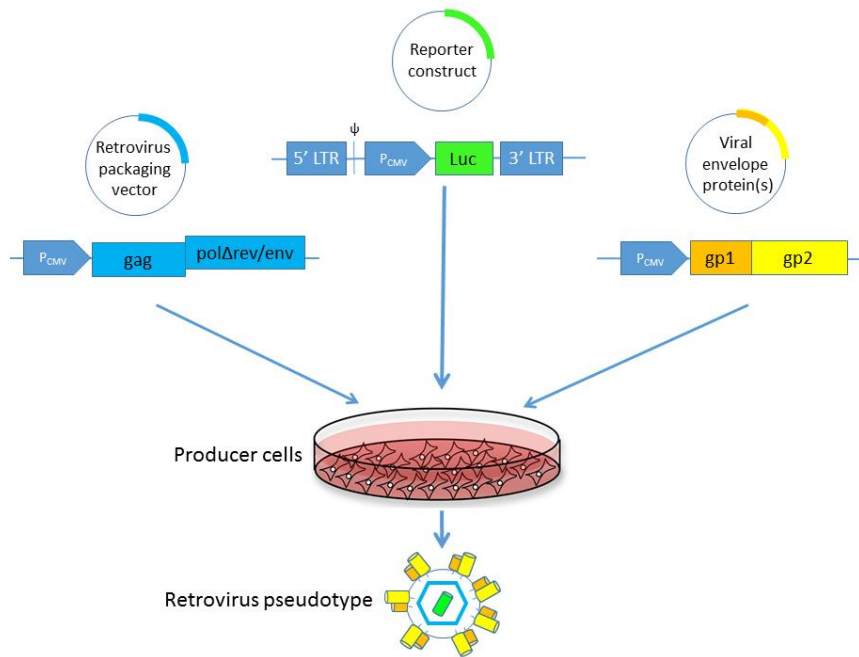
Figure 1.

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727 Figure 2.

728

729   Highlighted references:

730

731   14. Whitt 2010

732   This methods paper comprehensively describes the generation of pseudotyped viruses on the
733   vesicular stomatitis virus backbone

734

735   19. Urbanowicz 2016

736   This research article described the highly effective optimisation of retrovirus pseudotype generation
737   using a dilution matrix of the expression plasmids.

738

739   22. Landau 1991

740   This research described the phenomenon of bi-valent in vivo pseudotyping (expression of envelope
741   glycoproteins from more than one virus on individual virions) and the subsequent alteration of entry
742   phenotypes of the cells co-infected with HTLV and HIV.

743

744   38. Urbanowicz 2016

745   In this article pseudotyped retroviruses were used to map the entry phenotypes of Ebola
746   glycoprotein mutations identified during the recent West African outbreak and their differential
747   abilities to enter human and bat cell lines.

748

749   50. Deng 1996

750   Identification of the HIV-1 co-receptor CC-CKR-5 (aka CCR5) had a major impact on the field of HIV-1
751   research

752

753   63. Bartosch 2003

754   Identification of the HCV co-receptor SR-B1 had a major impact on the field of HCV research

755

756   97. Meredith 2016

757   Description of NTCP as entry factor for HBV entry has opened significant new avenues of research
758   and potential treatment areas for this major global disease.

759

760   106. King 2016

761   This Perspective describes the principles of pseudotype generation on retrovirus backbones,

762   discusses the merits of different systems and provides examples of the known methods for

763   opotimising retrovirus PV production and infection assays

764